ADVERSE EFFECTS OF BONE MORPHOGENIC PROTEIN-2 DURING OSSEOINTEGRATION

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ADVERSE EFFECTS OF BONE MORPHOGENIC PROTEIN-2

DURING OSSEOINTEGRATION

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LIST OF SYMBOLS AND ABBREVIATIONS

BMP2	Bone morphogenic protein 2
IL	Interleukin
MSC	Mesenchymal stem cells
NHOst	Normal human osteoblasts
Ti	Titanium
TCPS	Tissue culture polystyrene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TAK1	Transforming growth factor β–activated kinase 1
TAB	TAK1-binding protein
shNOG-MG63	Silenced Noggin MG63 cell line
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

SUMMARY

Modifications of biomaterial surface properties are employed to increase osteoblast differentiation and bone formation. Microtextured metallic surfaces promote osteoblast differentiation and high surface energy- achieved by controlling surface hydrocarbon contamination- increases osteoblast differentiation and peri-implant bone formation. Recombinant human bone morphogenic protein 2 (BMP2) is approved to induce bone formation in a number of applications. It is used clinically in combination with biomaterials to improve peri-implant bone formation and osseointegration. The amount of BMP2 that is required is large and inflammatory (swelling/seroma) and bone-related (ectopic bone/bone resorption) complications have been reported after BMP2 treatment. The aim of this study was to examine potential deleterious effects of BMP2 on the inflammatory environment and apoptosis of osteoblasts.

Surface roughness and energy decreased pro-inflammatory interleukins and increased anti-inflammatory interleukins. In contrast, BMP2 abolished the surface effect, increasing pro-inflammatory interleukin (IL) 6, IL8, and IL17 in a surface roughness-dependent fashion and decreasing anti-inflammatory IL10 on rough surfaces. 5Z-7-Oxozeaenol and Dorsomorphin, but not H-8, blocked the effect of BMP2 on IL1A expression. There was an increase in expression of IL6 when treated with BMP2 for the control and H-8 groups, but both 5Z-7-Oxozeaenol and Dorsomorphin blocked the effect. Both 5Z-7-Oxozeaenol and H-8 blocked the effect of BMP2 on IL10 expression.

BMP2 treatment had little effect on apoptosis in human mesenchymal stem cells (MSCs). Exogenous BMP2 had no effect on TUNEL. Caspase-3 activity was

increased only at 200ng/ml BMP2. BAX/BCL2 decreased in MSCs treated with 50 and 100ng/ml BMP2. In contrast, BMP2 increased caspase-3 activity and TUNEL at all doses in normal human osteoblasts (NHOst). BAX/BCL2 increased in NHOst treated with BMP2 in a dose-dependent manner. Cells treated with 200 ng/ml BMP2 had an 8-fold increase in BAX/BCL2 expression in comparison with untreated cells. Similarly, BMP2 increased DNA fragmentation in NHOst cells. The BMP2-induced increase in DNA fragmentation was eliminated by 5-Z7-Oxozeaenol and Dorsomorphin.

The results suggest that while surface features modulate an initial controlled inflammatory response, the addition of BMP2 induces a pro-inflammatory response. The effect of BMP2 on apoptosis depends on cell maturation state, inducing apoptosis in committed osteoblasts. BMP2 together with microtextured orthopaedic and dental implants may increase inflammation and possibly delay bone formation. Dose, location, and delivery strategies are important considerations in BMP2 as a therapeutic and must be optimized to minimize complications.

CHAPTER 1

INTRODUCTION

Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) were first described in 1965 by Marshall Urist as the active component of demineralized bone matrix able to induce ectopic bone formation. BMPs are members of the TGF-β growth factor superfamily (1). BMPs are generated by proteolytic cleavage of the transcribed preproproteins (2). After cleavage, mature BMPs are secreted from the cell and form homodimers or heterodimers to activate signaling (3, 4). BMPs transduce signals through membrane serine/threonine kinase receptors (5-7). These receptors, categorized as type I and type II receptors, have differential specificity for BMP ligands (8, 9). Dimers of type I receptors combine with dimers of type II receptors to transduce signaling. It has been demonstrated that the order of receptor recruitment and binding determines signaling (9). BMP binding to preformed complexes activates Smad signaling, while BMP-induced formation of heteromeric complexes activates non-canonical BMP signaling (9). Smads are intracellular signaling proteins that are categorized as either receptor-regulated Smads, common-partner Smad, or inhibitory Smads (5, 10). Type I BMP receptors phosphorylate Smad1/5/8. The activated Smad then complexes with common-partner Smad4, translocates to the nucleus, and initiates downstream signaling. In addition to this canonical Smad signaling, BMP signaling can also activate mitogen activated kinase signaling molecules (ERK1/2, p38, Jun N-terminal kinase) through activation of transforming growth factor β–activated kinase 1 (TAK1) and TAK1-binding protein (TAB) (6, 11, 12).

BMP signaling is required for multiple processes in embryonic and adult tissue formation and homeostasis (1, 13-15). BMP signaling controls mesoderm patterning, chondrogenesis and osteogenesis in skeletogenesis, cartilage and bone formation, and

limb development. In vitro, BMPs induce adipogenic, chondrogenic, and osteogenic differentiation of progenitor cells (16-18). BMP signaling also promotes patterning of craniofacial structures, and limb development. Programmed cell death, or apoptosis, is important in several of these processes. BMP2, BMP4, and BMP7 initiate apoptosis in the developing limb (19-21). While the effects of BMPs on apoptosis are clear, the controlling mechanisms in embryonic and adult processes remain to be elucidated.

Besides roles in development, BMP2 is essential in bone regeneration. In a mouse model, limb-specific deletion of BMP2 expression delayed endochondral bone formation and led to micro-fractures in animals by post-natal day 14 (22, 23). Recombinant BMP2 and BMP7 (OP-1) are approved by the Food and Drug Administration for use in bone formation (24). BMP2 is used in fracture repair, anterior lumbar spinal fusions, alveolar ridge augmentation, and sinus lifts. Additionally, there is interest in combining BMP2 with biomaterials to enhance bone formation around the implanted material. However, incidences of ectopic bone formation, swelling of soft tissue, and bone resorption have been reported with its use (25-27).

Host-biomaterial Interaction

Biomaterials are defined as "natural or synthetic materials used to direct, supplement, or replace functions of living tissues" (28). These materials can be categorized as ceramic, polymeric, metallic, or composite/hybrid materials. Because of their biocompatibility, durability, and strength, metals such as stainless steel, cobalt-chromium and alloys, and titanium and titanium alloys are frequently used for dental and orthopaedic biomaterial implants. The aim of these implants is direct apposition of bone to the biomaterial, termed osseointegration, while achieving maintenance and functional restoration of the existing bone. Titanium and titanium alloys possess material properties that make them advantageous for biomaterial applications. First, they spontaneously form

an oxide layer that provides corrosion resistance. Second, this oxide layer also provides biocompatibility to the implant. Finally, titanium is stronger than stainless steel, but much less dense, and has high fatigue resistance, making it appropriate in load-bearing orthopaedic and dental applications (29).

The interaction between the patient host tissue and the implant material determines the overall success of the implant. Although titanium is biocompatible, biological, physical, and chemical surface modifications have been employed to enhance osseointegration. Biological modifications such as tethering of adhesion motifs (RGD sequences), hydroxyapatite coatings, and bioactive proteins (including BMP2) have been investigated to encourage formation of bone around the implant. Physical and chemical modifications such as sandblasting, acid etching, oxidation, plasma spray, or combinations of these techniques, are applied to titanium biomaterials to alter surface chemistry, topography, and wettability.

The first event after implant placement is the attachment of blood and serum proteins to the implant surface. These interactions are regulated by material properties of the implant, including chemical composition, energy, and micro-nanotopography (30-32). The cells that attach to the implant surface do not directly interact with the material, but with this adsorbed protein layer (33, 34). Surface properties also control cell shape (35, 36), which can effect gene transcription and cell response (37). In vitro, sand-blasted/acid-etched titanium surfaces (SLA) or hydrophilic SLA (modSLA) induce osteoblasts maturation to a greater extent than cells cultured on smooth Ti or tissue culture polystyrene (TCPS) phenotype (36, 38, 39). In addition, osteoblasts cultured on SLA or modSLA secrete higher levels of autocrine or paracrine factures that regulate

osteogenesis (40), including prostaglandin E2, osteoprotegerin, transforming growth factor beta-1, Wnt5a, Dickkopf-1 and -2, and BMP2 (38, 41-45). Factors secreted by cells on microstructured Ti surfaces are also able to induce differentiation of mesenchymal stem cells in a co-culture model (46), indicating that paracrine regulation of distal cells is involved in osseointegration. Results of in vitro studies have been correlated with in vivo and clinical outcomes. SLA and modSLA implants have greater bone-to-implant contact and removal torque and faster healing than smooth Ti implants (47-52). However, whether surface properties modulate immune response is unclear.

After implant placement, the initial blood clot is remodeled by immune cells that mediate the wound healing process. Cytokines are pleotropic proteins that regulate the inflammatory response in an autocrine, paracrine, and endocrine fashion (53). Both innate and inflammatory responses can be triggered by biomaterial implantation. Inflammation typically resolves during the normal course of healing. However, if a chronic inflammatory environment is triggered, phagocytic immune cells can lead to structural and chemical implant damage and possibly result in implant failure (54, 55). Surface properties such as hydrophilicity (54) and the combination of roughness and hydrophilicity (56) affect macrophage and dendritic cell phenotype. This evidence suggests that surface properties can be tailored to affect immune response. However, it is not clear whether osteoblasts differentiating on the implant surface are also able to secrete factors that modulate the inflammatory microenvironment and induce rapid healing.

SPECIFIC AIMS

The **objective** of this study is to characterize the effect of BMP2 on regulation of inflammatory interleukin production and its relation to titanium surface roughness, and to determine the effect of BMP2 on osteoprogenitor and osteoblast apoptosis and the potential mechanism of action.

The overall **hypothesis** is that BMP2 will affect interleukin production and induce apoptosis in osteoblasts.

Aim 1: Determine the effect of BMP2 on regulation of pro- and anti-inflammatory interleukins and relation to implant surface roughness

Aim 1.1: Examine production of interleukins by osteoblasts cultured on microtextured titanium surfaces

Aim 1.2: Examine the effect of BMP2 addition on interleukin production

Aim 1.3: Determine BMP pathway involved in this effect using specific small molecule inhibitors of BMP signaling

Aim 2: Determine the effect of BMPs on osteoprogenitor and osteoblast apoptosis and the potential mechanism of action

Aim 2.1: Determine the effect of BMP2 on osteoprogenitor and osteoblast apoptosis

Aim 2.2: Determine the role of Noggin on osteoblast apoptosis

Aim 2.3: Determine pathway by which effect of BMP occurs

CHAPTER 2

INTRODUCTION

Biocompatibility of materials is an important consideration in the development of dental and orthopaedic implants, as this factor can greatly affect the success of the implant. Cells interact with the surface of the implant and these interactions dictate the response of the cells to the biomaterial and lead to osseointegration of the implant to the bone. Surface properties have been modified to increase osteoblast differentiation and bone formation. It has been shown that microtextured titanium surfaces promote osteoblast differentiation and that high surface energy, achieved by controlling surface hydrocarbon contamination, further increases osteoblast differentiation, maturation, and bone formation around implants (57, 58).

Peri-implant osteogenesis requires net new bone formation. While stimulatory effects on osteoblast maturation are important, control of osteoclastic remodeling is also critical. Several studies have shown that osteoblasts contribute to this process by producing higher levels of osteoprotegerin (OPG) (40, 59), a decoy receptor for RANK ligand, thereby potentially reducing recruitment and activation of osteoclasts (59, 60). Inflammation processes play a role as well, particularly during the acute inflammatory response triggered in the early periods after injury (61, 62). Because of these initial inflammatory events, mesenchymal stem cells (MSCs) are recruited to the site of injury and begin tissue repair (63-65). Regulation of bone formation and subsequent bone remodeling can also be adversely affected by inflammatory responses, however, causing a shift from bone formation towards osteoclastogenesis and bone resorption that could impair osseointegration or lead to implant failure (66-69).

In vivo studies indicate that inflammation plays a role in osseointegration of metallic implants (70-73). We recently reported that dendritic cell maturation is reduced on microtextured Ti implant surfaces with high wettability (56), indicating that immune cells are sensitive to biomaterial surface properties. However, production of inflammatory cytokines is not limited to immune cells. Osteoblasts produce abundant cytokines to regulate inflammation, which include interleukins (IL).

ILs can be classified as pro- or anti-inflammatory, although many can exhibit both types of behavior depending on the concentration. IL1 is one of the most well studied pro-inflammatory cytokines, existing in two isoforms (IL1α and IL1β) that function to activate macrophage and neutrophil responses (74, 75), promoting a T_H1 immune response and suppressing the T_H2 cell response. IL6, a pro-inflammatory cytokine that may also possess anti-inflammatory properties, stimulates B cells to produce immunoglobulins (76), promotes inflammation (77), and activates osteoclasts (78, 79). The cytokine IL7 regulates differentiation, proliferation, and survival of T lymphocytes (80). and studies have demonstrated that this pro-inflammatory factor plays an important role in inflammatory bone diseases (81), working together with other pro-inflammatory cytokines in the promotion of osteoclastogenesis (81), and activates target inflammatory genes such as chemokines, cytokines, and nitric oxide (82, 83). One of the most powerful anti-inflammatory cytokines, IL10 (84, 85), inhibits T_H1 cytokines, monocytes, macrophages, and neutrophils (86), modulating acute inflammation and promoting humoral T_H2 immune responses.

Interleukins work together with many other cytokines in a complex network that determines the overall inflammatory response of the host to an implanted biomaterial.

However, determining the host-material interaction becomes more complicated as exogenous factors are introduced to enhance bone apposition. Bone morphogenetic proteins (BMPs) induce bone formation in development (1, 87). Recombinant human BMP2 (rhBMP2) is used clinically to induce bone formation in hard-to-heal fractures and defects, and is now being used in combination with metallic biomaterials to induce regeneration of bone around implants (88-91). Although this growth factor has been demonstrated to increase osteogenesis in the lumbar spine (92, 93), inflammatory complications including seroma and osteolysis have been reported to occur (25, 26, 94-96). There are a number of possible reasons for this, but the local concentration of BMP2 may be a contributing factor (96).

While cytokine production is necessary for immune responses to eliminate foreign pathogens, an imbalanced pro-inflammatory response after biomaterial implantation can produce extreme inflammation and possibly compromise biomaterial success. Little is known about the effects of the surface properties of an implant and BMP2 on the inflammatory microenvironment created by osteoblasts during osseointegration. The aim of the current study was to determine whether pro-osteogenic surface properties modulate the inflammatory interleukin response of osteoblasts in vitro and determine the effect exogenous BMP2 has on this process.

MATERIALS AND METHODS

Cell Culture

Fifteen-millimeter Ti disks were prepared from 1mm thick sheets of grade 2 unalloyed Ti and supplied to us by Institut Straumann AG (Basel, Switzerland). The methods used to produce smooth pretreatment (PT, Ra=0.08μm), rough sandblasted acid

etched (SLA, Ra=3.22μm), and modified SLA (modSLA, Ra=3.22μm) have been reported previously (36, 97). Hydrophilicity of the surfaces was calculated using advancing contact angles [PT (95.8°), SLA (139.80°), and modSLA (~0°)].

Human MG63 osteoblast-like osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). These cells are a progenitor cell model of osteoblast differentiation used in biomaterial studies, and have been previously demonstrated to increase markers of osteoblast maturation in response to Ti substrates with micron- and submicron-scale roughness and high surface energy (98, 99). MG63 cells were plated on tissue culture polystyrene (TCPS) or Ti substrates at a density of 10,000 cells per cm^2. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were cultured at 37°C and 100% humidity.

Interleukin Expression

IL mRNA was measured in cells cultured as described on TCPS or Ti substrates. At confluence on TCPS, cells on all surfaces were incubated with fresh medium for 12h and RNA harvested using a TRIzol® (Invitrogen, Carlsbad, CA) extraction method following manufacturer's protocol. mRNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). RNA (500 ng) was amplified using reverse transcription (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Carlsbad, CA). Starting quantities of mRNA were determined using SybrGreen chemistry (Power SYBR® Green PCR Master Mix, Applied Biosystems) in a StepOne Plus imaging system (Applied Biosystems). MG63 cells grown on TCPS were used to generate a standard curve for each gene of interest and values for each sample

extrapolated. mRNA was measured for IL1A, IL1B, IL6, IL7, IL8, IL10, and IL17 (sequences in Table 1) using gene specific primers (MWG Operon, Huntsville, AL). Osteocalcin mRNA (OCN) was measured as an indicator of osteoblast differentiation. All genes are presented as normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 2.1. Primer sequences used in real-time qPCR analysis

mRNA	Sequence		Accession Number
IL1A	F	TTT GAC GAC GCA CTT GTA GC	NM_000575
	R	GCC ATG AAA TGA CTC CCT CTC	
IL1B	F	TGG CAG AAA GGG AAC AGA AAG G	NM_000576
	R	AAC AAA AGG GCT GGG GAT TGG	
IL6	F	GCA GAA AAC AAC CTG AAC CTT C	NM_000600
	R	ACC TCA AAC TCC AAA AGA CCA G	
IL7	F	TCT TCT TCT GTG CTG GAG ATG	NM_000880
	R	GGA CCT TGT TAT GCT GTT GC	
IL8	F	GAC ATA CTC CCA AAC CTT TCC AC	NM_000584
	R	AAA CCT CTC CAC AAC CCT CTG	
IL10	F	TTA TCT TGT CTC TGG GCT TGG	NM_000572
	R	GAA TGA AGT GGT TGG GGA ATG	
IL17A	F	TGG GGC AGA ACG AAC TTT AAG	NM_002190
	R	GGC GAA AAT GGT TAC GAT GTG	
MSX2	F	CCT CTC CCT CTC CAC GAA G	NM_002449.4
	R	TGC CTC CGC CTA CAG AAC	
OCN	F	GTG ACG AGT TGG CTG ACC	NM_199173
	R	TGG AGA GGA GCA GAA CTG G	
GAPDH	F	GCT CTC CAG AAC ATC ATC C	NM_002046.3
	R	TGC TTC ACC ACC TTC TTG	

Interleukin Protein Production

Cells were cultured as described on TCPS or Ti disks and at confluence on TCPS, cells on all surfaces were incubated with fresh medium for 24h. Cells were released from TCPS and Ti surfaces using two sequential incubations with 0.25% trypsin for 10 min at 37°C and counted using a cell counter (Z2 Particle counter, Beckman Coulter, Fullerton,

CA). Cellular alkaline phosphatase specific activity was measured to verify cell response to the microstructured Ti surfaces. Briefly, alkaline phosphatase specific activity (orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1) was determined in cell lysates using a colorimetric assay measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at 37°C (100). Levels of secreted cytokines IL1α, IL6, IL8, IL10, and IL17 were assayed in the conditioned medium using ELISA (R&D Systems DuoSet, Minneapolis, MN). Protein levels were normalized to total cell number.

To determine the effect of BMP2 on interleukin production, cells were cultured as described on TCPS or Ti substrates. At confluence on TCPS, cells were incubated with fresh medium ± 40ng/ml rhBMP2 for 24 hours. ELISA assays were used to quantify the pro-inflammatory and anti-inflammatory cytokines in the conditioned medium.

Effect of BMP Pathway on Interleukin Expression

BMP2 binds to tetramers of BMP receptors, initiating downstream signaling through canonical Smad signaling or non-canonical protein kinase A (PKA) or TAB/TAK1 signaling. To determine the BMP pathway involved in inflammatory cytokine expression, specific BMP pathway inhibitors were used. (5Z) -7-Oxozeaenol, a TAK1 inhibitor (101, 102), Dorsomorphin, an inhibitor of type I BMP receptors (ALK2, ALK3, ALK6) that prevents Smad phosphorylation (103-105), and H-8, a protein kinase A inhibitor (106-108), were purchased from EMD Chemicals (Gibbstown, NJ). Cells were cultured on modSLA substrates as described above. Cells were pre-incubated for 2 hours with control media, 100 nM (5Z)-7-Oxozeaenol, 10 nM Dorsomorphin, or 1 μM H-8 followed by treatment with inhibitor ± 40 ng/ml rhBMP2 for 12 hours. RNA was harvested and expression quantified as described above.

Statistical Analysis

Data presented are from one of two sets of experiments, with comparable results. Each data point is the mean ± SEM of six independent cultures. Data were analyzed by analysis of variance and significant differences between groups determined using Bonferroni's modification of the Student's t-test. $P<0.05$ was considered to be significant.

RESULTS

Effect of Surface Roughness on Interleukin Expression

mRNAs for osteocalcin increased in a surface roughness and energy dependent manner (Fig. 2.1A), confirming increased cell maturation. mRNAs for anti-inflammatory IL10 increased with increasing surface roughness and energy, with a 3-fold increase on modSLA in comparison to TCPS or PT (Fig. 2.1B).

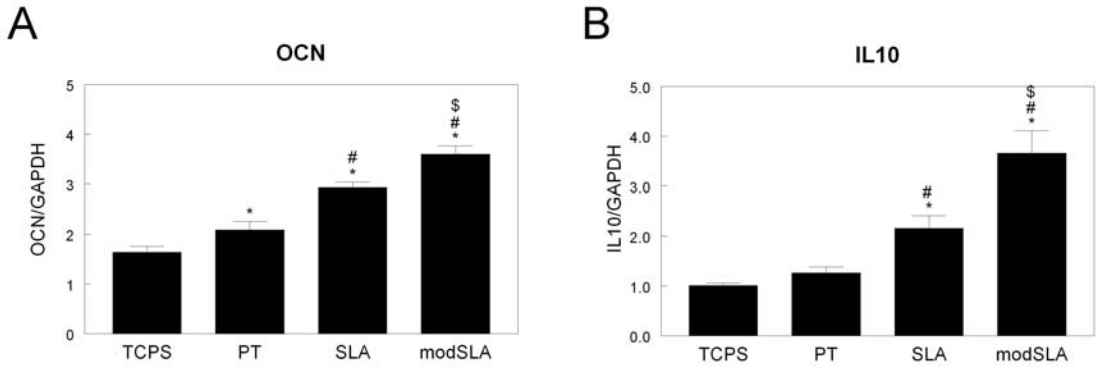

Figure 2.1. Effect of surface microstructure and energy on anti-inflammatory mRNA levels. MG63 cells were cultured on TCPS or Ti surfaces and expression of OCN (A) and IL10 (B) measured by real-time qPCR. *$p<0.05$, versus TCPS; # $p<0.05$, versus PT; \$ $p<0.05$, versus SLA.

Expression of pro-inflammatory cytokines was also regulated by surface roughness and energy. IL1A expression was increased on rough surfaces (Fig. 2.2A). However, while IL1B expression was 1.5-times higher on the rough SLA surface than on TCPS or PT, levels on modSLA were similar to PT (Fig. 2.2B). IL6 expression decreased on SLA in comparison to TCPS or PT, but was lowest on modSLA (Fig. 2.2C). IL7

expression decreased on all titanium surfaces in comparison to TCPS (Fig. 2.2D). Levels were similar in cells grown on PT or modSLA surfaces, but were highest on SLA. Expression of IL8 (Fig. 2.2E) decreased on SLA and modSLA surfaces in comparison to TCPS or PT, with the lowest expression on modSLA substrates. However, mRNAs for IL17 decreased on rough SLA surfaces and were further lowered by culture on modSLA (Fig. 2.2F).

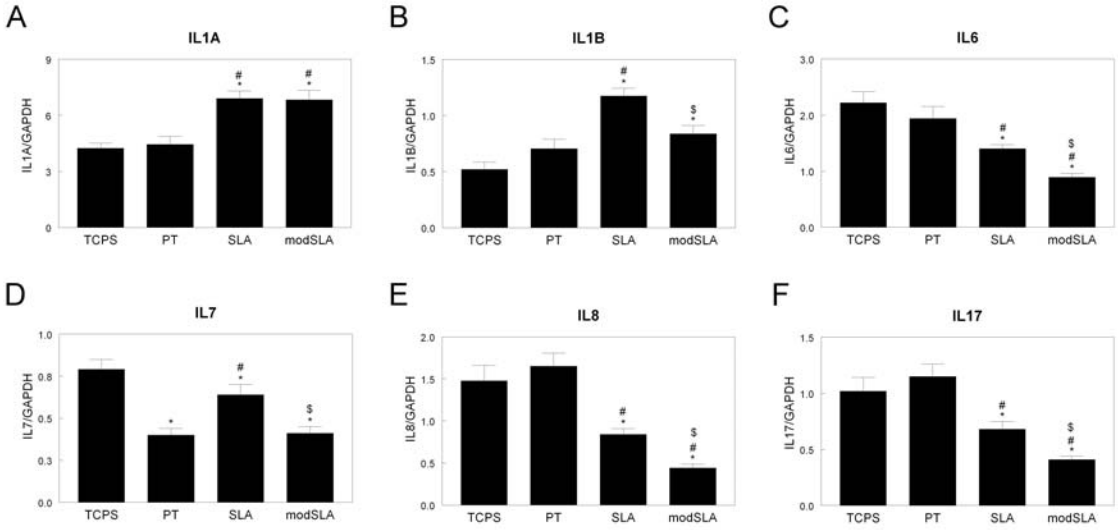

Figure 2.2. Effect of surface microstructure and energy on pro-inflammatory interleukin mRNA levels in MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces and expression of IL1A (A), IL1B (B), IL6 (C), IL7 (D), IL8 (E), and IL17 (F) measured by real-time qPCR. *p<0.05, versus TCPS; # p<0.05, versus PT; $ p<0.05, versus SLA.

Effect of Surface Roughness on Interleukin Production

Cell number was lower on titanium surfaces than on TCPS, with further decreases on rough (SLA) and rough/hydrophilic surfaces (Fig. 2.3A). Alkaline phosphatase specific activity (Fig. 2.3B) increased in a roughness and surface energy-dependent manner (TCPS < PT < SLA < modSLA). These results confirm cell response to these materials as demonstrated previously (36, 45).

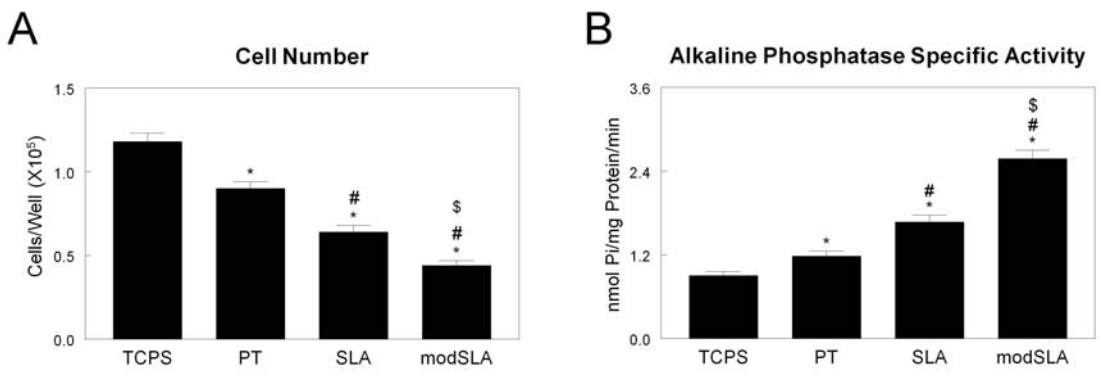

Figure 2.3. Effect of surface microstructure and energy on osteogenic differentiation of MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces to confluence and cell number (A) and alkaline phosphatase specific activity (B) measured. *p<0.05, versus TCPS; # p<0.05, versus PT; $ p<0.05, versus SLA.

Cells on Ti had higher IL10 production than TCPS, but modSLA induced highest production of the anti-inflammatory IL10 (Fig. 2.4A). Surface roughness increased secreted IL1α by 100% in comparison to TCPS or PT surfaces, but levels on modSLA surfaces were 50% higher than SLA (Fig. 2.4B). Levels of secreted IL6 and decreased in a roughness and surface energy-dependent manner, with the lowest levels produced on modSLA surfaces (Fig. 2.4C). IL8 production was similar on TCPS and PT surfaces, but levels were lower on SLA, and lowest on modSLA (Fig. 2.4D). Secreted IL17 was lower on PT and SLA surfaces than on TCPS; however, levels were lowest on modSLA (Fig. 2.4E).

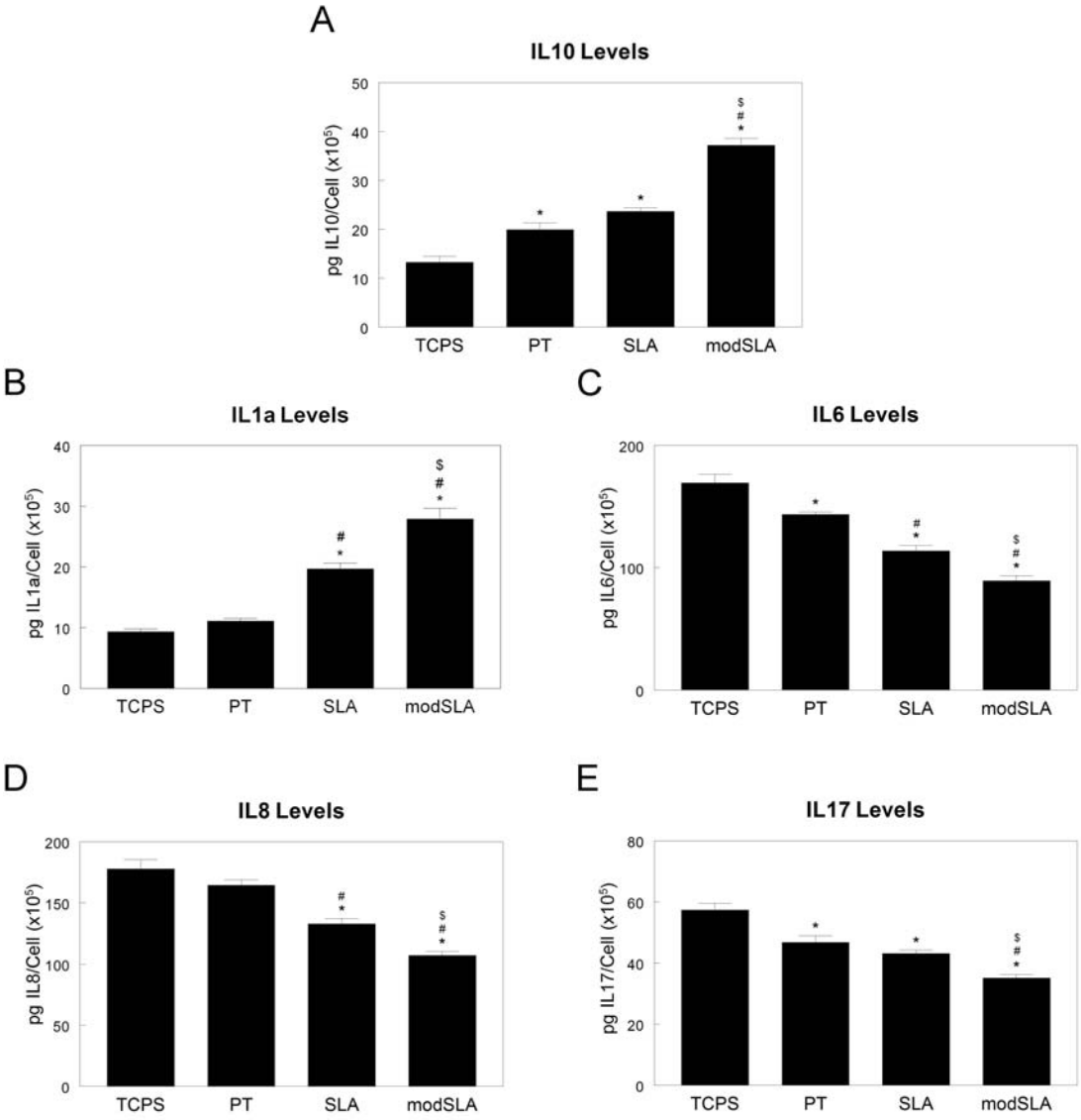

Figure 2.4. Effect of surface microstructure and energy on interleukin production by MG63 cells. MG63 cells were cultured on TCPS or Ti surfaces and production of anti-inflammatory IL10 (A) or pro-inflammatory IL1α (B), IL6 (C), IL8 (D), and IL17 (E) measured in the conditioned media. *$p<0.05$, versus TCPS; # $p<0.05$, versus PT; \$ $p<0.05$, versus SLA.

Effect of Bone Morphogenetic Protein-2 on Interleukin Production

BMP2 treatment had no effect on cell number on the surfaces examined (Fig. 2.5A). Alkaline phosphatase specific activity was higher in BMP2 treated cells on SLA surfaces than in untreated cells (Fig. 2.5B).

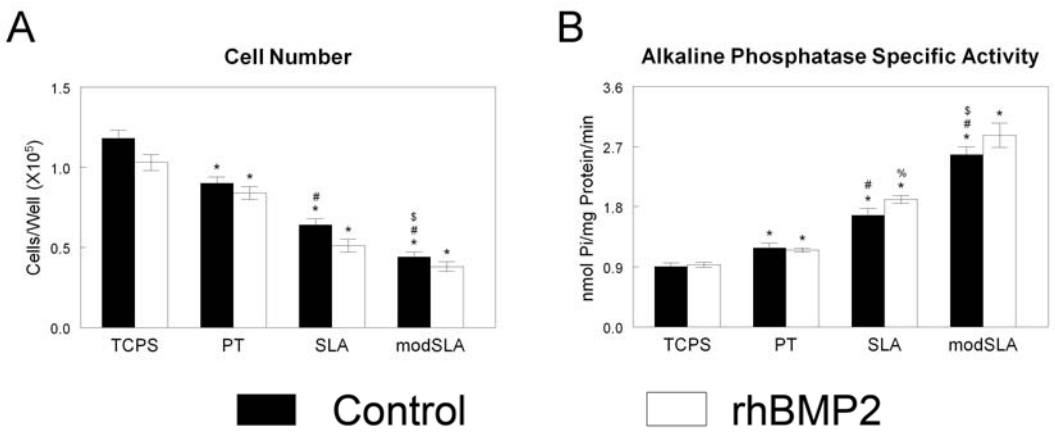

Figure 2.5. Effect of BMP2 on MG63 differentiation on microstructured Ti surfaces. MG63 cells were cultured on TCPS or Ti surfaces. At confluence, cells were treated with medium (control) or 40 ng/ml rhBMP2 for 24 hours and cell number (A) or alkaline phosphatase specific activity (B) measured. *p<0.05, versus TCPS; # p<0.05, versus PT; $ p<0.05, versus SLA; %p<0.05, versus control.

The increase in anti-inflammatory IL10 seen in cells culture on hydrophilic rough surfaces was abolished by BMP2 treatment (Fig. 2.6A). However, the increase in IL1α production by cells on rough surfaces was enhanced by BMP2 treatment (Fig. 2.6B). While culture on rough or high-energy rough surfaces decreased production of IL6 and IL8, treatment with BMP2 reversed this effect (Fig. 2.6C, 2.6D). Addition of BMP2 to cells on rough Ti caused a 100% increase in IL17 production in comparison to untreated cells (Fig. 2.6E).

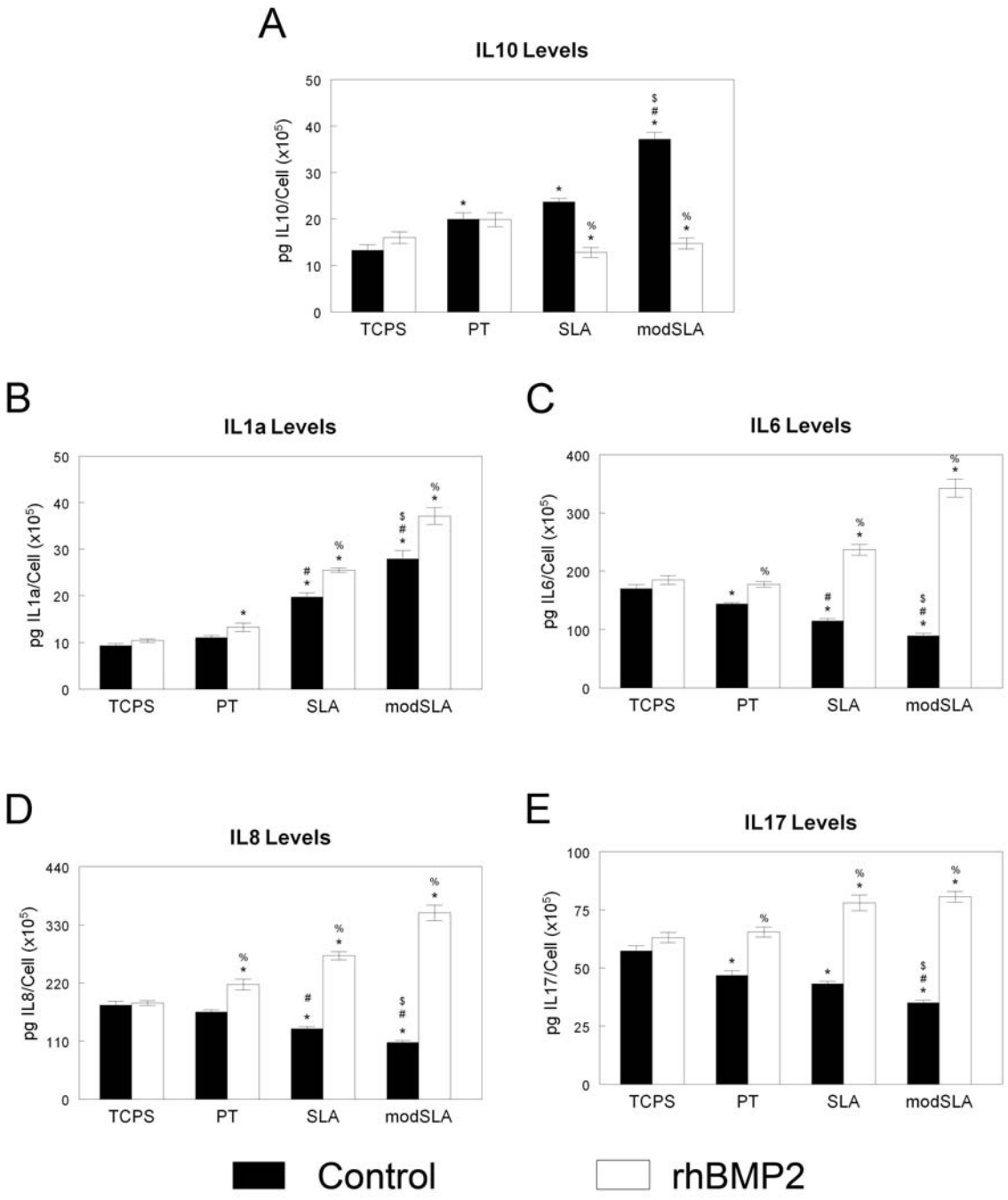

Figure 2.6. Effect of BMP2 on inflammatory interleukin expression in MG63 cells cultured on microstructured Ti surfaces. MG63 cells were cultured on TCPS or Ti surfaces. At confluence, cells were treated with medium (control) or 40 ng/ml rhBMP2 for 12 hours and mRNA levels of IL10 (A), IL1α (B), IL6 (C), IL8 (D) measured. *p<0.05, versus TCPS; # p<0.05, versus PT; $ p<0.05, versus SLA; %p<0.05, versus control.

Mechanism of BMP2 Effect

Treatment with BMP2 increased expression of MSX2, a factor that is transcribed after induction of BMP2 signaling (Fig. 2.7A). Treatment with Dorsomorphin, but not 5Z-7-Oxozeaenol or H8, abolished this effect. All three inhibitors abolished the effect of BMP2 treatment on expression of IL10 (Fig. 2.7B). Increased expression of IL1A, IL6, and IL17 after BMP2 treatment was blocked with 5Z-7-Oxozeaenol and Dorsomorphin, but not H8 (Fig. 2.7B, 2.7C, 2.7F). BMP2 induced upregulation of IL8 expression was inhibited by both all three inhibitors (Fig. 2.7E).

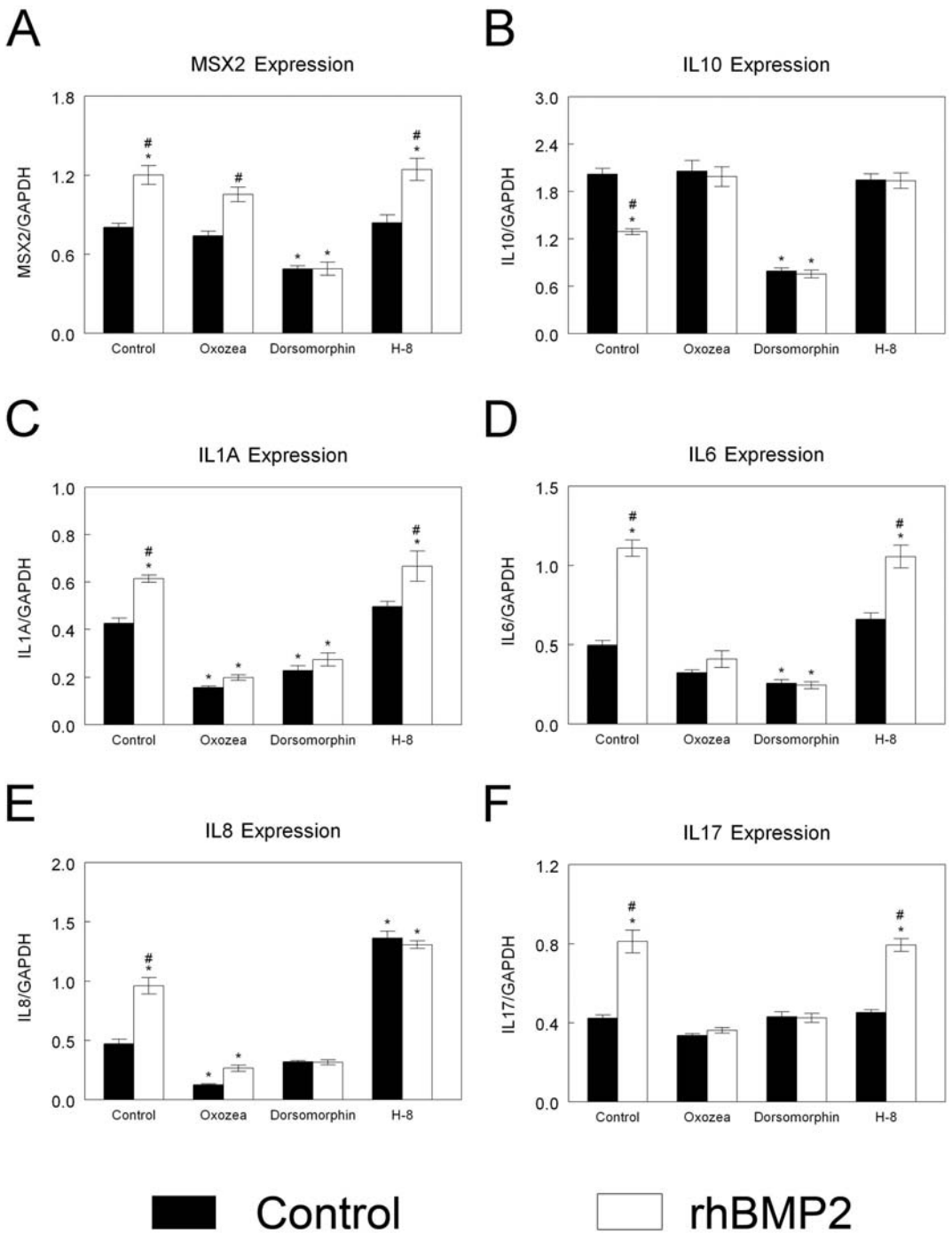

Figure 2.7. BMP pathway modulation of inflammatory microenvironment produced by MG63 cells on microstructured Ti surfaces. MG63 cells were cultured on modSLA surfaces. Cells were treated with inhibitors targeting specific BMP signaling pathway components TAB/TAK (5Z-7-Oxozeaenol), Smad (Dorsomorphin), or PKA (H-8) in the presence or absence of 40 ng/ml rhBMP2. Levels of MSX2 (A), IL10 (B), IL1A (C), IL6 (D), IL8 (E), and IL17 (F) mRNA were measured 12 hours after treatment. *$p<0.05$, versus control; #$p<0.05$, BMP2 treatment versus untreated cells.

DISCUSSION

An inflammatory response is necessary for osseointegration (73), but must occur in a coordinated series of events to prevent skewing the response from bone formation to bone resorption. In the current study, we examined the effect of Ti surface properties to modulate inflammatory interleukin production by osteoblasts. The results show that both surface topography and energy affect interleukin gene expression and protein levels in MG63 cells. Moreover, these effects are altered by rhBMP2 through TAB/TAK signaling.

MG63 cells had higher IL1A and IL1B expression on rough and hydrophilic rough Ti surfaces and produced higher levels of these proteins. Both IL1α and IL1β are pro-inflammatory cytokines; however, they may play other roles as well. MSCs treated with IL1β exhibit an osteoblastic phenotype, including formation of Alizarin red positive nodules (109). Moreover, delivery of IL1β during the first 72 hours after fracture sped healing in an *in vivo* model (110). In addition, IL1β expression is increased in bone during fracture healing (111). Thus, an increase in these cytokines at low concentration is consistent with enhanced bone healing.

In contrast to the stimulatory effects of the microtextured surfaces on osteoblast production of IL1α and IL1β, the cells on these surfaces exhibited reduced expression and secretion of other inflammatory cytokines, such as IL6. IL6 induces bone resorption by increasing osteoclast activity (79, 112) and levels of this interleukin are increased in aseptic loosening (113-115). In vivo, the reduction in IL6 may help increase peri-implant bone formation by reducing osteoclast activity.

Similarly, rough surfaces and high surface energy significantly reduced IL8 and IL17 expression and levels. IL8 is a powerful recruiter of neutrophils and is present in high levels in areas of polymorphonuclear infiltration (116) and in prostate cancer-induced osteolytic lesions (117). IL17 activates matrix metalloproteinases in MC3T3-E1 osteoblasts, causing increased matrix degradation (81). There is evidence that IL17 modulates the OPG-RANKL balance, increasing osteoclastogenesis and inducing bone remodeling (118, 119).

Importantly, the anti-inflammatory cytokine IL10 was increased on rougher surfaces, an effect more robust with increased surface energy. IL10 inhibits NF-κB and suppresses production of pro-inflammatory cytokines by monocytes and macrophages (120, 121). This suggests that rougher surfaces, particularly on modSLA where hydrocarbon deposition is controlled, promote an anti-inflammatory response. The results demonstrate that cells on microstructure Ti surfaces secrete factors to modulate the bone formation process, and this effect is enhanced on rough, high-energy surfaces.

In addition to direct effects on bone formation, it has also been shown that surface properties can affect other parameters that may affect implant success, including response of immune system cells to the biomaterial. Hydrophilic rough surfaces were found to support an immature dendritic phenotype while smooth surfaces enhanced dendritic cell maturation (56). Macrophages cultured on the same hydrophilic rough surfaces expressed lower levels of pro-inflammatory cytokines than cells on smooth substrates (122). Other studies have demonstrated that macrophages regulate inflammatory cytokines in response to changes in surface chemistry and topography (123-125). Taken together, surface properties directly control the inflammatory microenvironment by created by osteoblasts

and activation of immune cells, suggesting that this immuno-modulation may contribute to faster healing seen clinically in modSLA implants.

In our study, administration of BMP2 to osteoblasts cultured on microstructured Ti implants increased pro-inflammatory, pro-osteoclastogenic interleukins and decreased the anti-inflammatory IL10. It is important to note that the effect of BMP2 was not observed on TCPS, a material commonly used for *in vitro* studies, but that BMP2 had a strong synergistic effect on roughness and energy. Interestingly, treatment with BMP2 induced the opposite effect in cytokine profile in comparison to surface roughness and energy alone. BMPs are important in bone formation, and induce chondrogenic and osteogenic commitment of progenitor cells (87).

Recombinant human BMP2 is one of two clinically available BMPs approved for use. While this morphogen has been demonstrated to induce bone formation, there have been reports of adverse effects including ectopic bone formation, osteolysis, and seroma formation (25, 26, 126). In animal models of inflammation, soft tissue inflammation was seen as early as 3 hours after subcutaneous implantation of rhBMP2, an effect dependent on protein dose (127, 128). The same study found increases in levels of secreted IL6. Studies have demonstrated that BMP2 upregulates IL6 in normal human osteoblasts, confirming the present results (129). Interestingly, in the dose used in our study, the increase in pro-inflammatory interleukin secretion came with only small gains in osteoblast maturation.

In our study, the TAB/TAK1 inhibitor (5Z)-7-Oxozeaenol blocked BMP2 induced IL6 expression. TAK1 has been demonstrated to mediate IL-6 expression during cyclic stress in osteoblasts (130). In addition, TAK1 silencing decreases the pro-inflammatory environment in a mouse inflammatory model (131). The inflammatory environment

induced by high doses of BMP2 is likely due to the ability of BMP2 to activate this signaling pathway.

CONCLUSIONS

Taken together, the results suggest surface roughness and energy modulate the initial inflammatory response, increasing anti-inflammatory interleukins and reducing pro-inflammatory interleukins. Surfaces known to induce osteoblast differentiation also modulate interleukin production. The control of interleukins production and other inflammatory processes by surface features may have a major role in bone healing and osseointegration. However, BMP2 treatment during orthopaedic and dental titanium implant insertion may produce adverse effects through TAB/TAK signaling by reversing the surface effect on the inflammation process, increasing pro-inflammatory interleukins, which may possibly delay the bone formation process.

CHAPTER 3

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) super family (7), and a subset of BMPs possess the ability to induce bone and cartilage formation and enhance osteogenesis (132, 133). BMP2 is used clinically to induce bone formation in challenging bone defects in orthopaedics and dentistry (134). However, incidences of complications have been reported in orthopaedic applications including soft tissue swelling, ectopic bone formation, and even resorption of adjacent vertebral bodies (25, 135).

BMP2 must form homodimers or heterodimers to activate the BMP signaling pathway (4). The dimer then binds to a complex of four transmembrane receptors, consisting of two BMP type I receptors and two BMP type II receptors (7, 136), and one of three signaling pathways is initiated depending on the conformation of receptors upon ligand binding (9). In the first, BMP type I receptors phosphorylate Smad1, Smad5, or Smad8. This Smad then associates with Smad4 and the complex translocates to the nucleus and activates transcription of downstream genes (6, 137). Alternatively, p38 or JNK mitogen-activated protein kinase (MAPK) signaling can be initiated by TGF-β1 activated kinase-1 (TAK-1) and TAK1 binding protein (TAB) (138, 139). Finally, protein kinase A (PKA) can be activated (140, 141), potentially leading to ERK1/2 MAPK activation. BMP signaling is regulated extracellularly by soluble inhibitors such as Noggin, Gremlin, Cerberus, and Chordin, which physically bind to BMPs, preventing their dimerization or receptor binding, inhibiting transduction of downstream signaling (2, 142).

BMPs induce cartilage and bone formation both during embryonic development and post-natally (13, 14) and they regulate other developmental and adult processes including cell growth and differentiation (15, 143, 144), as well as apoptosis (145-148).

Control of apoptosis during development ensures proper tissue morphogenesis. For example, BMP2 regulates fibroblast growth factor signaling to induce apoptosis in the apical ectodermal ridge during limb formation (149). In the chick limb bud, both BMP2 and BMP4 are expressed in the anterior, posterior, and interdigital necrotic zones prior to apoptosis (150). However, there is limited evidence about the effects of BMP2 on apoptosis in adult osteoprogenitor cells or in committed osteoblasts.

Given the effects seen following the clinical use, the aim of this study was to characterize the effect of BMP2 on apoptosis of osteoprogenitor cells and osteoblasts, and the role of soluble BMP antagonist Noggin on this process, and lastly the BMP pathway used to induce apoptosis.

MATERIALS AND METHODS

Cell Culture

The effect of BMP2 on apoptosis was examined in osteoprogenitor cells (MSCs), immature osteoblasts (MG63 cells), and mature osteoblasts (NHOst cells). Human bone marrow-derived mesenchymal stem cells (MSCs) were purchased from Lonza Biosciences (Walkersville, MD) and cultured in Mesenchymal Stem Cell Growth Media (MGSCGM, Lonza Biosciences). Human MG63 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modification of Eagle's Medium (cellgro®, MediaTech, Manassas, VA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Gibco). Normal human osteoblasts (NHOst cells, Lonza) were cultured in Osteoblast Growth Media (Lonza). For all experiments, cells were plated at a density of 10,000 cells/cm^2 and cultured at 37°C, 5% CO_2, and 100% humidity. Media were changed 24 hours after plating and every 48 hours thereafter until cells reached confluence.

Recombinant human BMP2 (R&D Systems, Minneapolis, MN) was reconstituted in sterile 4 mM HCl containing 0.1% bovine serum albumin to a stock concentration of 100 µg/ml. The stock was diluted to final concentrations in culture medium. At confluence, cells were treated with 50, 100, or 200 ng/ml BMP2 unless noted otherwise.

Proteome Profiler Array

The effect of BMP2 on osteoblast apoptosis was investigated using a Human Apoptosis Proteome Profiler Array (R&D Systems), which examined changes in 35 apoptosis-related proteins. Confluent cultures of NHOst cells were treated with 200 ng/ml BMP2 for 6 hours. After incubation, cells were lysed and analyzed by modified Western blot. Membranes were imaged by chemiluminescence (VersaDoc, Bio-Rad, Hercules, CA) and changes in proteins determined by densitometry (QuantyOne, Bio-Rad).

Caspase-3

Cells were treated with BMP2 as described for 6 hours and assayed for caspase-3 activity using a commercial kit (R&D Systems) following manufacturer's instructions. Briefly, cell monolayers were lysed in cold lysis buffer for 10 minutes at 4°C, and the cell lysates centrifuged at 10,000*g* for 1 minute. The resulting supernatant was combined with 2x reaction buffer and DEVD-pNA substrate and incubated at 37°C for 2 hours. Absorbance at 405 nm was determined using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA).

BAX/BCL2 mRNA Levels

Cells were treated with BMP2 for 12 hours. RNA was isolated using TriZOL® (Invitrogen, Carlsbad, CA) and was quantified (Nanodrop Spectrophotometer, Thermo Scientific, Waltham, MA). 250 ng of RNA was used to synthesize cDNA libraries (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Carlsbad, CA). Levels of mRNA were quantified with real-time quantitative PCR (StepOnePlus, Applied

Biosystems) using gene-specific primers (Table 1) and *Power* SybrGreen® Master Mix (Applied Biosystems). Starting mRNA quantities were determined by standard curve method. Levels of mRNA are presented as a ratio of BAX to BCL2.

Table 3.1: Primer sequences used in real-time qPCR analysis

Gene	Primer Sequence	
BAX	F	GACGAACTGGACAGTAACATGG
	R	AAAGTAGAAAAGGGCGACAACC
BCL-2	F	CTGTTTGATTTCTCCTGGCTGTCTC
	R	TCTACTGCTTTAGTGAACCTTTTGC
NOG	QuantiTect Primer Assay, QT00210833	
GAPDH	F	GCTCTCCAGAACATCATCC
	R	TGCTTCACCACCTTCTTG

Total p53 Levels

Total p53 levels were determined in cells 24 hours after treatment using a sandwich ELISA (Human Total p53 DuoSet® IC, R&D Systems). After treatment, monolayers were rinsed with PBS and lysed [PBS containing 1 mM EDTA, 0.5% Triton^{TM} X-100, 10 mM NaF, 150 mM NaCl, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 3 µg/ml aprotinin]. Lysates were centrifuged at 2000*g* for 5 minutes and the supernatant assayed for total p53 following manufacturer's instructions. Results were normalized to total protein content (Pierce 600nm Protein Assay, Thermo Scientific).

DNA Fragmentation

TUNEL Assay

DNA fragmentation was assessed by colorimetric terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL, TiterTACS™ in situ

Microplate TUNEL Assay, R&D Systems). Confluent cultures were treated for 24 hours. Media were removed, and cells were fixed with 3.7% sucrose-buffered formaldehyde and processed following manufacturer's instructions. Absorbance at 450 nm was determined using a microplate reader.

Radiometric Assay

At 60% confluence, cells were incubated with 1 μCi/ml ^{3}H-thymidine (Perkin Elmer, Waltham, MA). At confluence, cultures were treated for 24 hours with BMP2. Cells were lysed [10mM Tris-HCl, 1mM EDTA, 0.2% Triton X-100] and subjected to three freeze-thaw cycles. Intact DNA was separated from fragmented DNA by ultracentrifugation at 13,000*g* for 15 minutes. Intact DNA (pellet) and fragmented DNA (supernatant) were counted by liquid scintillation counting (Beckman Coulter). Results are presented as percent fragmented DNA/total DNA.

Noggin Expression

Since soluble inhibitors regulate the actions of BMPs, we wanted to determine the effect of BMP2 treatment on mRNA levels of NOG, one of the most powerful inhibitors of BMPs. Cells were treated with BMP2 as described. Levels of mRNA for NOG were determined after 12 hours as described above and are presented as normalized to mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Noggin Silencing

shNOG-MG63 cells were examined to determine the role of Noggin in osteoblast apoptosis. MG63 cells silenced for Noggin (shNOG-MG63) were generated using lentiviral shRNA transduction particles (NM_005450, TRCN0000058563, Mission®, Sigma Aldrich, St. Louis, MO). MG63 cells were plated at 20,000 cells/cm^2 and cultured overnight in full medium. Particles were added to the cells at a multiplicity of infection of

7.5 in full medium supplemented with 8 μg/ml hexadimethrine bromide (Sigma Aldrich) and incubated for 18 hours. After incubation, transduced cells were selected with full medium containing 0.25 μg/ml puromycin. The resulting cell line had a 70% reduction in NOG mRNA levels as determined by real-time qPCR and 72% reduction in secreted Noggin as determined by ELISA (data not shown).

BMP2 Signaling Pathway

Confluent cultures of NHOst cells were pre-treated for one hour with inhibitors of BMP2-dependent signaling pathways. 5Z-7-oxozeaenol (100 nM) was used to inhibit TAB/TAK1 signaling (101); dorsomorphin (10 nM) was used to inhibit Smad signaling (104, 105); H-8 (1 μM) was used to inhibit PKA signaling (151). All inhibitors were purchased from EMD Chemicals (San Diego, CA). BMP2 was added to the media containing the inhibitor to a final concentration of 200 ng/ml. Cells were incubated and assayed as described above.

Statistical Analysis

Data are presented as mean ± SEM of six independent cultures per variable. Data were first examined by analysis of variance (ANOVA) and significant differences between groups were determined using Bonferroni's modification of Student's t-test. $P<0.05$ was considered to be significant.

RESULTS

The results of the proteome profiler array showed that of the 35 apoptosis-related proteins assessed (Fig. 3.1A), 9 proteins were significantly higher and 5 proteins were lower in BMP2-treated NHOst cells than in untreated cells (Fig. 3.1B). Levels of pro-apoptotic proteins such as Bad, Bax, pro-caspase-3, and phosphorylated p53 (Ser 15)

were increased more than 20% in NHOst cells treated with BMP2 than in control cells (Fig. 3.1B). Anti-apoptotic markers Bcl-x, Omi, Livin, and XIAP were 25% lower in BMP2-treated NHOst cells than in untreated cultures (Fig. 3.1B).

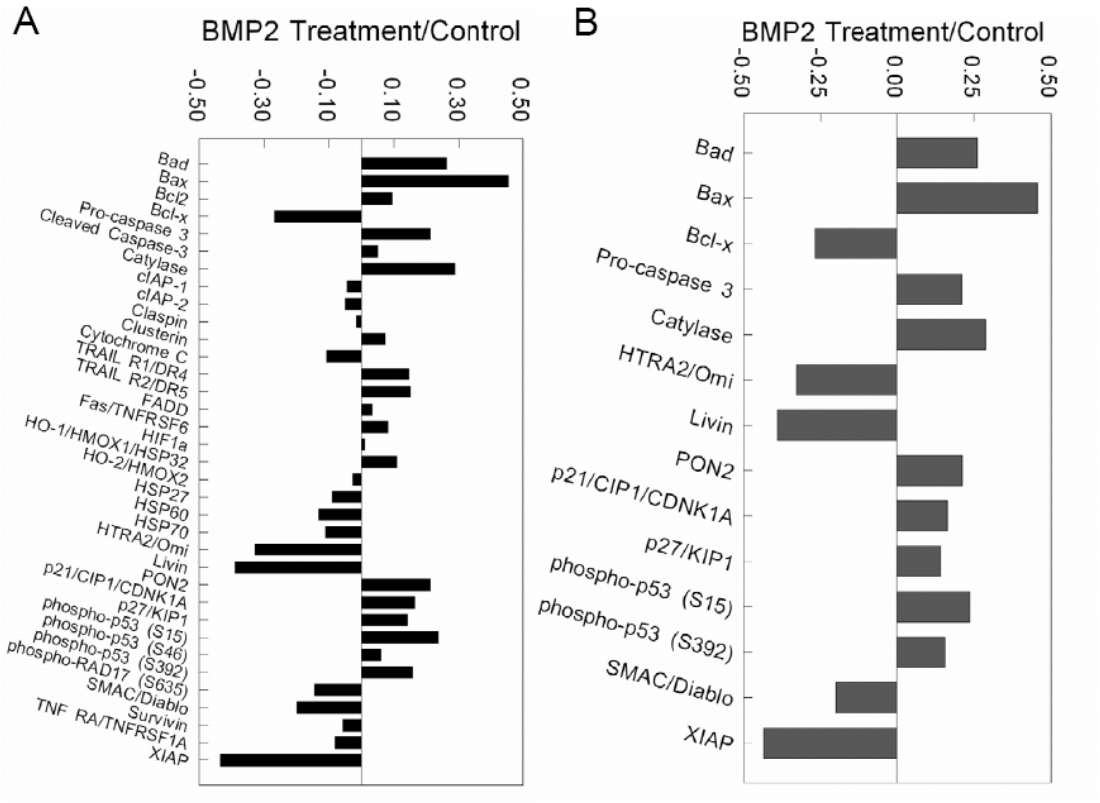

Figure 3.1. Effect of BMP2 treatment on apoptotic proteins in NHOst cells. Confluent cultures of NHOst cells were treated for 6 hours with BMP2. Cell lysates were analyzed by modified Western blot for levels of 32 proteins known to be involved in apoptosis and densitometry analysis performed (A), and proteins with more than 15% changes after BMP2 treatment identified (B).

The effect of BMP2 on apoptosis varied with cell type. MSCs treated with 200 ng/ml BMP2 exhibited a small increase in caspase-3, with no effect at lower doses (Fig. 3.2A). Addition of BMP2 increased total p53 at all concentrations (Fig. 3.2C), but had no effect on BAX/BCL2 (Fig. 3.2B) or TUNEL (Fig. 3.2D). In MG63 cells, addition of 100

or 200 ng/ml BMP2 increased caspase-3 (Fig. 3.3A). However, BAX/BCL2 (Fig. 3.3B) and p53 (Fig. 3.3C) were only increased by 200 ng/ml BMP2. BMP2 had no effect on TUNEL in MG63 cells at the doses examined (Fig. 3.3D).

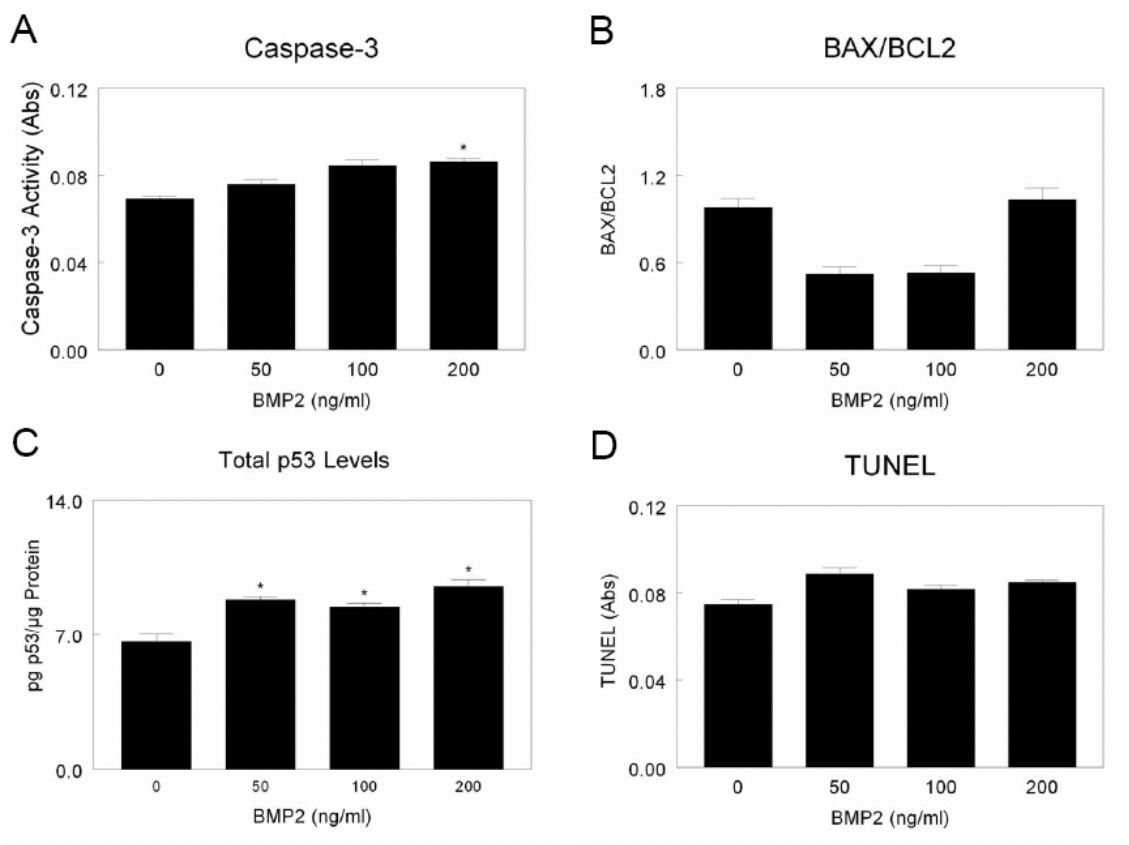

Figure 3.2. Effect of BMP2 on mesenchymal stem cell apoptosis. MSCs were cultured until they reached confluence. Cells were treated with 50, 100, or 200 ng/ml BMP2 and Caspase-3 (A), BAX/BCL2 (B), total p53 (C), and TUNEL (D) measured. *p< 0.05, versus control; #p< 0.05, versus 50 ng/ml BMP2; $p< 0.05, versus 100 ng/ml BMP2.

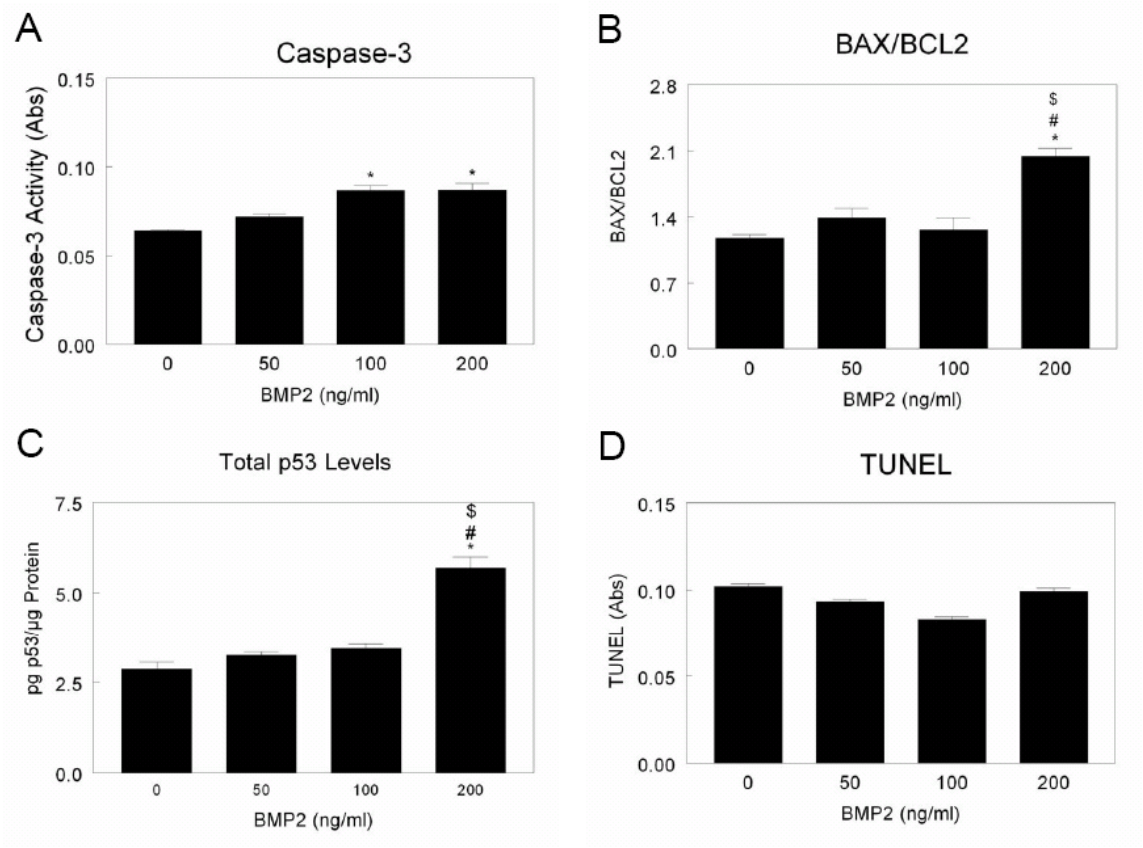

Figure 3.3. Effect of BMP2 on MG63 cell apoptosis. MG63 cells were cultured until they reached confluence. Cells were treated with 50, 100, or 200 ng/ml BMP2 and Caspase-3 (A), BAX/BCL2 (B), total p53 (C), and TUNEL (D) measured. *p< 0.05, versus control; #p< 0.05, versus 50 ng/ml BMP2; $p< 0.05, versus 100 ng/ml BMP2.

In contrast, BMP2 significantly increased caspase-3 in NHOst cells at all doses examined (Fig. 3.4A). BAX/BCL2 was increased after BMP2 treatment in a dose-dependent manner, with a 7-fold increase after 200 ng/ml BMP treatment (Fig. 3.4B). Levels of p53 were higher in 100 ng/ml and 200 ng/ml treated cultures than control cultures (Fig. 3.4C). TUNEL was increased in cultures treated with 200 ng/ml BMP2 (Fig. 3.4D), and this was confirmed by a 50% increase in percent fragmented DNA (data not shown).

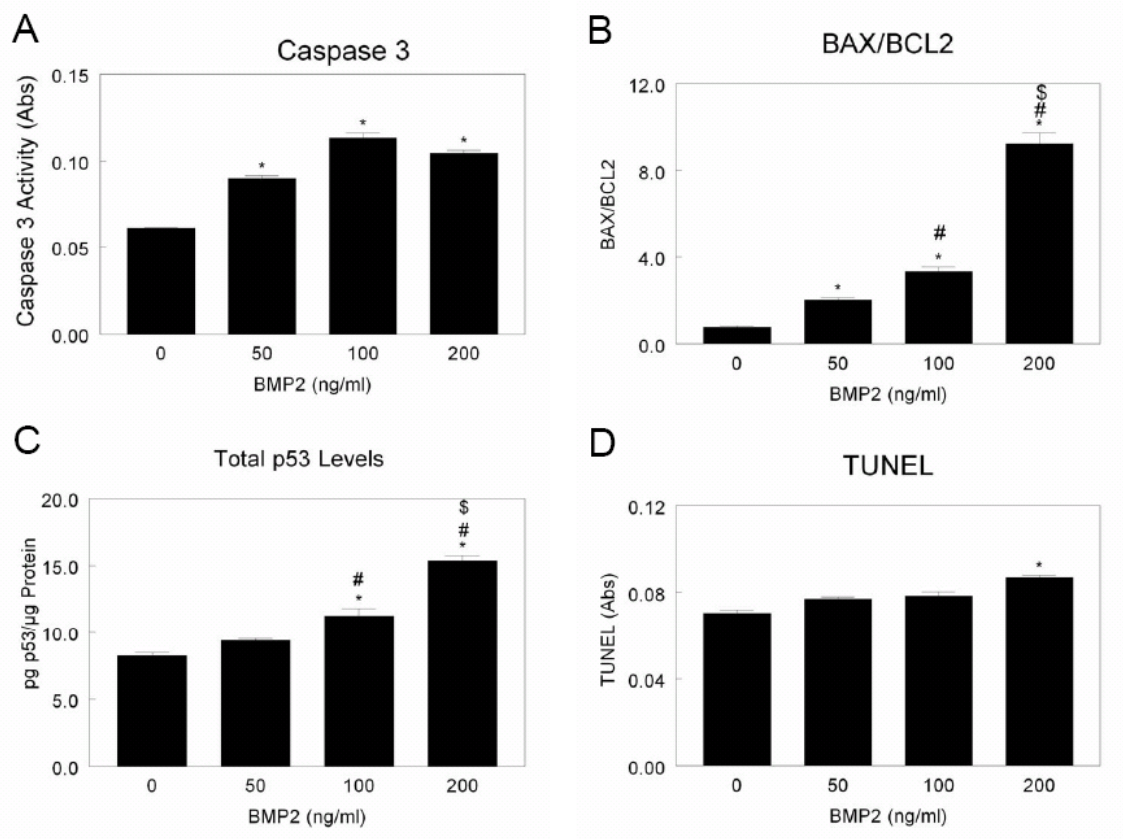

Figure 3.4. Effect of BMP2 on normal human osteoblast apoptosis. NHOst cells were cultured until they reached confluence. Cells were treated with 50, 100, or 200 ng/ml BMP2 and Caspase-3 (A), BAX/BCL2 (B), total p53 (C), and TUNEL (D) measured. *p< 0.05, versus control; #p< 0.05, versus 50 ng/ml BMP2; $p< 0.05, versus 100 ng/ml BMP2.

Noggin expression was regulated by BMP2 in all cell types examined. In MSCs, NOG was 1.5-fold higher than control after BMP2 treatment, but decreased with increasing BMP2 dose (Fig. 3.5A). In MG63 cells, treatment with BMP2 induced a greater than 2-fold increase in NOG mRNA levels, an effect that increased with increased BMP2 dose (Fig. 3.5B). A similar effect was seen in NHOst, with a 3-fold increase in NOG mRNA levels after 200 ng/ml BMP2 (Fig. 3.5C).

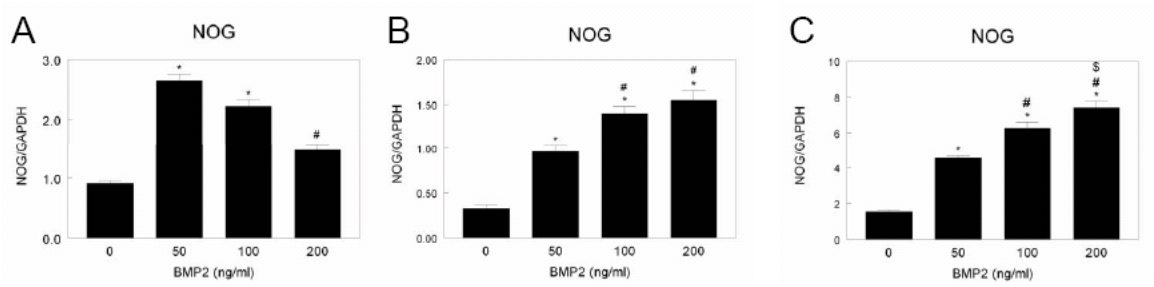

Figure 3.5. Effect of BMP2 treatment on NOG mRNA levels. Confluent cultures of MSCs (A), MG63 cells (B), and NHOst (C) were treated for 12 hours with 50, 100, or 200 ng/ml BMP2 and levels of mRNA for NOG measured by real-time qPCR. *p< 0.05, versus control; #p< 0.05, versus 50 ng/ml BMP2; $p< 0.05, versus 100 ng/ml BMP2.

Reduced Noggin expression resulted in increased expression of apoptotic markers and increased apoptosis. shNOG-MG63 cells had higher levels of apoptotic markers than wild type MG63 cells throughout the culture period. Wild type MG63 cells exhibited time-dependent increases in MTT activity (Fig. 3.6A). In contrast, shNOG-MG63 cells had increased MTT levels until day 5, when cells underwent apoptosis and MTT was reduced to 20% of levels seen in control (Fig. 3.6A). Caspase-3 was higher in shNOG-MG63 cells at days 2, 3, and 4 than in wild type MG63 cells (Fig. 3.6B). BAX/BCL2 was also 100% higher in shNOG-MG63 cells at day 2 and 4 than in MG63 cells (Fig. 3.6C). shNOG-MG63 cells had higher TUNEL than MG63 cells at all times examined (Fig. 3.6D). TUNEL was 100% higher in shNOG-MG63 cells at day 2 and 200% higher at day 3 and 4 in comparison to MG63 cells (Fig. 6D).

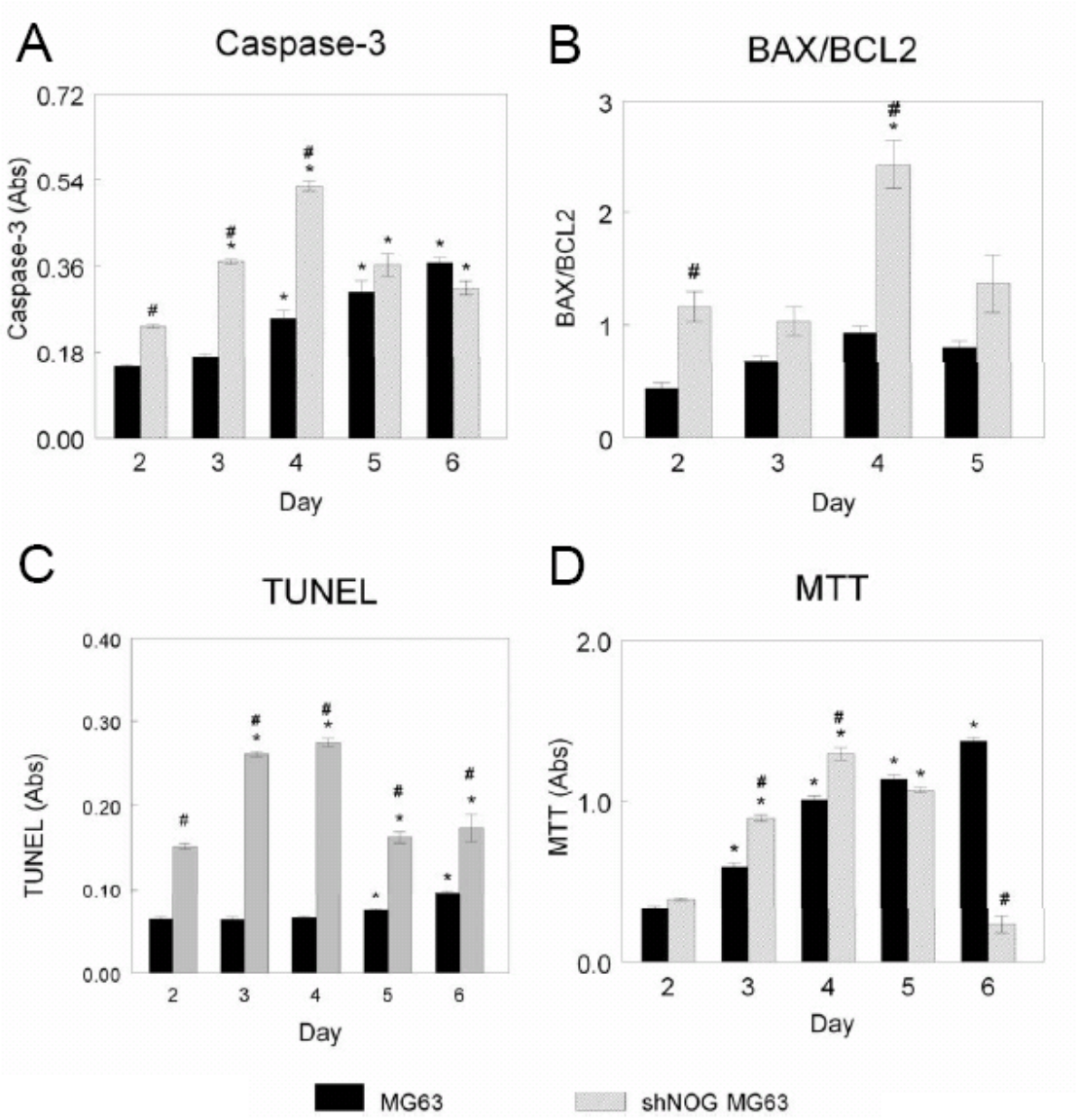

Figure 3.6. Effect of NOG silencing on MG63 cell apoptosis. MG63 and shNOG-MG63 cells were harvested at various time points after plating. Caspase-3 (A), BAX/BCL2 (B), TUNEL (C), and MTT (D) were assessed. *p< 0.05, versus day 2; #p< 0.05, versus MG63 cells.

The increased apoptosis in shNOG-MG63 cells was enhanced by BMP2. MG63 cells treated with 200 ng/ml BMP2 had a 25% increase in caspase-3; shNOG-MG63 cells had a higher baseline level of caspase-3, which was increased 25% by addition of BMP2 (Fig. 3.7A). Likewise, BAX/BCL2 increased in MG63 cells treated with BMP2. Baseline BAX/BCL was higher in shNOG-MG63 cells and was synergistically increased by exogenous BMP2 (Fig. 3.7B).

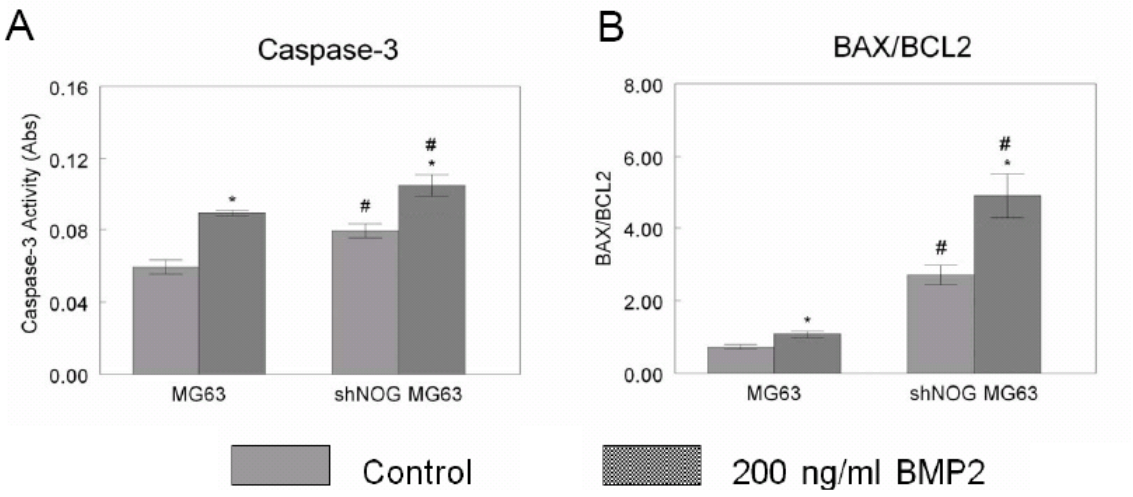

Figure 3.7. Effect of NOG silencing on BMP2-induced MG63 cell apoptosis. MG63 and shNOG-MG63 cells were treated with 200 ng/ml BMP2. Caspase-3 (A) and BAX/BCL2 (B) were assessed. *p< 0.05, versus control; #p< 0.05, versus MG63 cells.

The effect of BMP2 on apoptosis was via a Smad-dependent signaling pathway. In NHOst cells, the stimulatory effect of 200 ng/ml BMP2 on caspase-3 activity was blocked by dorsomorphin, but not by 5Z-7-oxozeaenol or H-8 (Fig. 3.8A). Likewise, only dorsomorphin blocked the BMP2-induced increase in BAX/BCL2 (Fig. 3.8B). However, increased DNA fragmentation after BMP2 treatment was inhibited by both dorsomorphin and 5Z-7-oxozeaenol (Fig. 3.8C), indicating that TAB/TAK1 signaling also played a role.

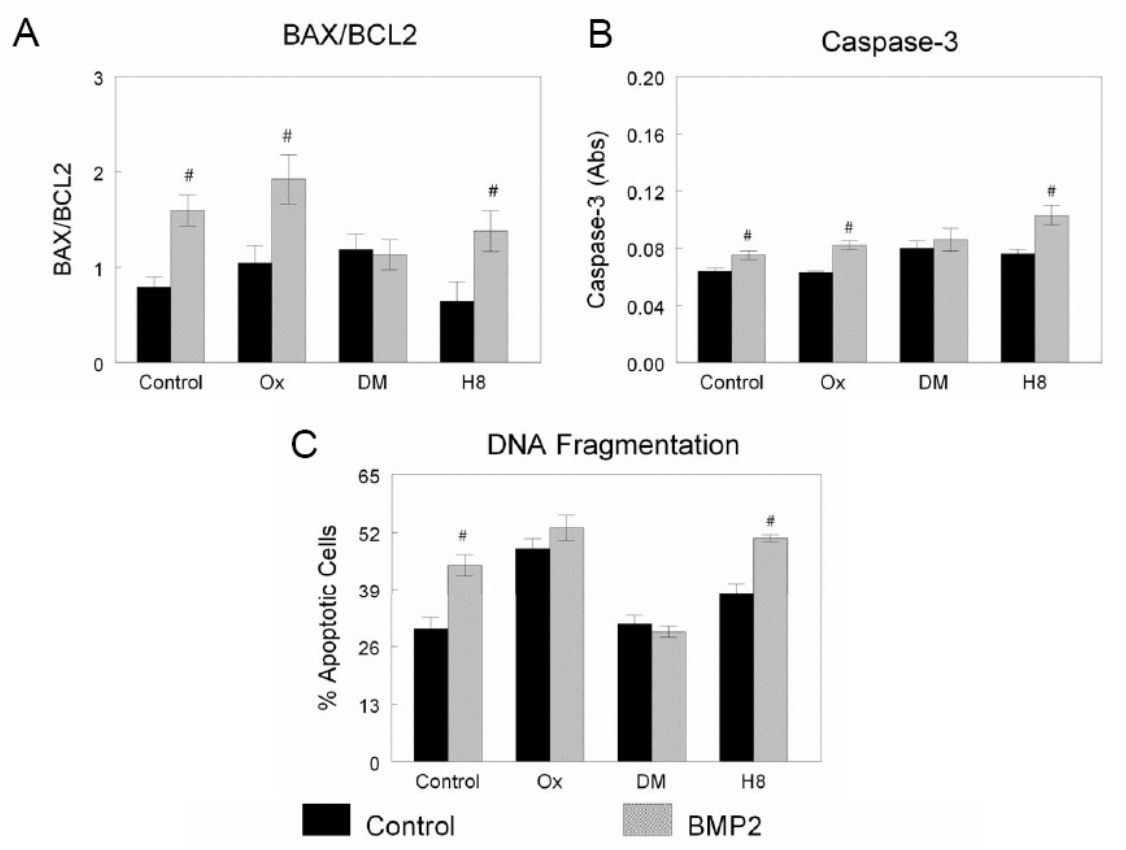

Figure 3.8. Effect of BMP pathway inhibition on BMP2-induced NHOst apoptosis. Confluent cultures of NHOst were pre-treated with inhibitors to TAB1/TAK (5Z-7-Oxozeaenol, Ox), Smad (Dorsomorphin, DM), or PKA (H8) before treatment with 200 ng/ml BMP2. Caspase-3 (A), BAX/BCL2 (B), and DNA fragmentation (C) were measured. #p< 0.05, versus untreated cells.

DISCUSSION

This study demonstrates that BMP2 modulates apoptosis in a cell-dependent manner. In MSCs, treatment with this morphogen had only small effects on apoptotic signaling. The immature osteoblast MG63 cells were sensitive to treatment with BMP2, but only at the highest dose used in our study. However, in NHOst cells, BMP2 induced apoptosis in a dose-dependent manner, an important fact given the exceedingly high doses used clinically.

BMP2 had little effect on apoptosis in MSCs in our study. BMPs are known to induce stem cell differentiation into chondrocytes, osteoblasts, and adipocytes in a dose-dependent manner (152). Moreover, several studies have indicated that BMP signaling is also required for maintenance of the stem cell niche and MSC survival (153, 154). This evidence suggests that BMPs affect maintenance and differentiation, but not apoptosis, in MSCs, in agreement with the results of our study.

Differential regulation of BMP2 effects were seen in cells committed to the osteoblast lineage. BMP2 increased BAX/BCL2, caspase-3, and TUNEL in immature osteoblast-like MG63 cells, confirming results using other immature osteoblast cell lines (148, 155). However, this effect was only at the highest dose tested. In committed osteoblasts, the effect was more robust, and occurred at lower doses. These observations suggest that the effect of BMP2 on osteoblast apoptosis is dependent on maturation state. In addition to up-regulating apoptotic markers, BMP2 down-regulated anti-apoptotic markers. In NHOst, anti-apoptotic Bcl-x was lower in cells treated with BMP2 than in untreated cells, confirming results seen in myeloma cells (147) and gastric cancer cells (146). Taken together, this emphasizes that the effect of BMP2 on cell apoptosis is conserved among cells of diverse origins, particularly those that are terminally differentiated.

BMPs are tightly regulated intracellularly by Smads (156) and extracellularly by diverse families of soluble inhibitors, including Cerberus, Twisted gastrulation, Chordin, Crossveinless, and Noggin (142). The present study demonstrates that Noggin, a powerful inhibitor of BMP signaling, was regulated by treatment with BMP2, an effect was dependent on cell type, and that silencing Noggin resulted in cell apoptosis. Our

results are in agreement with a number of studies in developmental biology. Loss of function Noggin transgenic mice present increased apoptosis during palatogenesis (157). Overexpression of Noggin in transgenic mice reduced apoptosis in eyelid epithelium (158) and during limb development (21). Noggin has also been found to regulate apoptosis during neural crest development (159). Interestingly, it has also been shown that the BMP inhibitor Sclerostin can also induce apoptosis in MSCs (160) and that administration of Gremlin during chick limb development inhibits apoptosis (161). In a clinical study, it was suggested that mRNA levels of BMP inhibitors during fracture healing might predict whether the facture will result in a non-union (64, 162, 163). These results demonstrate the complex regulation of BMPs and their inhibitors are critical in determining the final signaling outcome in tissue formation, regeneration, and homeostasis.

While it is clear that BMP signaling regulates cell survival, the pathways by which this occurs are unclear. In our study, inhibitors to Smad signaling blocked BMP-induced apoptosis in NHOst cells. Smad signaling also modulated apoptosis in colon cancer cells (164), but there is conflicting evidence on the pathway involved in osteoblast apoptosis. It has been suggested that this effect occurs through PKC-dependent, Smad-independent signaling (148); however this study used dominant negative Smad1 expression to establish this and did not examine the contributions of Smad 5 or 8. Here, we used dorsomorphin, which inhibits the ability of the BMP receptor to phosphorylate Smad1/5/8. Dorsomorphin is commonly used in the literature to inhibit canonical Smad-BMP signaling, particularly during zebrafish embryogenesis (103, 165) but also during mammalian processes (104, 166). Several studies have reported that dorsomorphin (and

its analog LDN193189) may inhibit pathways other than BMP signaling (165, 167). However, the dose of dorsomorphin shown to inhibit these pathways in C2C12 cells was higher (500 nM) than in our study (168). There were no off-target effects demonstrated with 5 or 50 nM dorsomorphin, suggesting that the 10 nM dose used in this study may not induce off-target effects.

CONCLUSIONS

Taken together, the results suggest that the effect of BMP2 treatment on osteoblast and progenitor apoptosis depends on the maturation state of the cell. While in progenitor cells BMP2 have minimal effects on cell apoptosis, in mature osteoblasts these potent factors induce apoptosis, especially at higher doses. The data suggest that in progenitor cells BMP2 acts more as a differentiation factor than an apoptogen and that in terminally differentiated cells, BMP2 induces apoptosis. It is possible that in clinical cases where complications arise with BMP2 administration, the osteogenic induction effect in MSCs is less robust than the apoptosis induced in mature osteoblasts, resulting in osteolysis.

CHAPTER 4

CONCLUSIONS

The objective of this study was to characterize the effect of BMP2 on regulation of inflammatory interleukin production and its relation to titanium implant surface roughness, and to determine the effect of BMP2 on osteoprogenitor and osteoblast apoptosis and the potential mechanism of action. The results of our study confirm the hypothesis that BMP2 affects interleukin production and induces apoptosis in osteoblasts, and elucidate the mechanisms of the complications seen clinically.

The data demonstrate that material surface properties such as roughness and hydrophilicity are able to regulate osteoblast interleukin production, increasing anti-inflammatory interleukins and reducing pro-inflammatory interleukins. These same surface features are known to induce osteoblast differentiation. The control of interleukin production by surface features may contribute to the shorter healing time seen with these same implants clinically, and demonstrate that interleukins play a major role in bone healing and osseointegration. However, co-administration of BMP2 with titanium implant insertion may produce adverse effects through TAB/TAK signaling by reversing the surface effect on the inflammation process, increasing pro-inflammatory interleukins. This deregulation of the inflammatory response may possibly delay bone formation.

The effects on existing bone seen during clinical use of BMP2 may be a result on the differential effect of BMP2 on osteoprogenitor and osteoblast apoptosis. The results of this study indicate that the effect of BMP2 on progenitor and osteoblast apoptosis depends on the maturation state of the cell. In progenitor cells, BMP2 has minimal effects on cell apoptosis. However, in mature osteoblasts this morphogen induces apoptosis, an

effect dependent on dose. It is possible that in clinical cases where complications arise with BMP2 administration, the osteogenic induction effect in MSCs is less robust than the apoptosis induced in mature osteoblasts, resulting in osteolysis. Dose, location, and delivery strategies are important considerations in BMP2 as a therapeutic and must be optimized to minimize complications.

REFERENCES

[1] Wan M, Cao X. BMP signaling in skeletal development. Biochem Biophys Res Commun. 2005;328(3):651-7.

[2] Rider CC, Mulloy B. Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists. Biochem J. 2010;429(1):1-12.

[3] Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, et al. Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci U S A. 1990;87(6):2220-4.

[4] Guo J, Wu G. The signaling and functions of heterodimeric bone morphogenetic proteins. Cytokine Growth Factor Rev. 2012;23(1-2):61-7.

[5] Massague J. TGF-beta signal transduction. Annual review of biochemistry. 1998;67:753-91.

[6] Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. J Biochem. 2010;147(1):35-51.

[7] Miyazono K, Maeda S, Imamura T. BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine & Growth Factor Reviews. 2005;16(3):251-63.

[8] Yin H, Yeh LC, Hinck AP, Lee JC. Characterization of ligand-binding properties of the human BMP type II receptor extracellular domain. J Mol Biol. 2008;378(1):191-203.

[9] Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, et al. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem. 2002;277(7):5330-8.

[10] Zwijsen A, Verschueren K, Huylebroeck D. New intracellular components of bone morphogenetic protein/Smad signaling cascades. FEBS Lett. 2003;546(1):133-9.

[11] Nohe A, Keating E, Knaus P, Petersen NO. Signal transduction of bone morphogenetic protein receptors. Cell Signal. 2004;16(3):291-9.

[12] Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F. p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. Exp Cell Res. 1999;250(2):351-63.

[13] Cao X, Chen D. The BMP signaling and in vivo bone formation. Gene. 2005;357(1):1-8.

[14] Nie X, Luukko K, Kettunen P. BMP signalling in craniofacial development. Int J Dev Biol. 2006;50(6):511-21.

[15] Reddi AH. BMPs: from bone morphogenetic proteins to body morphogenetic proteins. Cytokine Growth Factor Rev. 2005;16(3):249-50.

[16] Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev. 2000;21(4):393-411.

[17] Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev. 2003;24(2):218-35.

[18] Song C, Guo Z, Ma Q, Chen Z, Liu Z, Jia H, et al. Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. Biochem Biophys Res Commun. 2003;308(3):458-62.

[19] Bastida MF, Delgado MD, Wang B, Fallon JF, Fernandez-Teran M, Ros MA. Levels of Gli3 repressor correlate with Bmp4 expression and apoptosis during limb development. Dev Dyn. 2004;231(1):148-60.

[20] Zuzarte-Luis V, Montero JA, Rodriguez-Leon J, Merino R, Rodriguez-Rey JC, Hurle JM. A new role for BMP5 during limb development acting through the synergic activation of Smad and MAPK pathways. Dev Biol. 2004;272(1):39-52.

[21] Guha U, Gomes WA, Kobayashi T, Pestell RG, Kessler JA. In vivo evidence that BMP signaling is necessary for apoptosis in the mouse limb. Dev Biol. 2002;249(1):108-20.

[22] Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, et al. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet. 2006;38(12):1424-9.

[23] Taylor D, Hazenberg JG, Lee TC. Living with cracks: damage and repair in human bone. Nature materials. 2007;6(4):263-8.

[24] Benglis D, Wang MY, Levi AD. A comprehensive review of the safety profile of bone morphogenetic protein in spine surgery. Neurosurgery. 2008;62(5 Suppl 2):ONS423-31; discussion ONS31.

[25] Smoljanovic T, Bojanic I, Delimar D. Adverse effects of posterior lumbar interbody fusion using rhBMP-2. European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the

European Section of the Cervical Spine Research Society. 2009;18(6):920-3; author reply 4.

[26] Oetgen ME, Richards BS. Complications associated with the use of bone morphogenetic protein in pediatric patients. Journal of pediatric orthopedics. 2010;30(2):192-8.

[27] Boraiah S, Paul O, Hawkes D, Wickham M, Lorich DG. Complications of recombinant human BMP-2 for treating complex tibial plateau fractures: a preliminary report. Clin Orthop Relat Res. 2009;467(12):3257-62.

[28] Black J. Biological performance of materials : fundamentals of biocompatibility. 2nd ed. New York: Dekker; 1992. viii, 390 p. p.

[29] Niinomi M. Biologically and Mechanically Biocompatible Titanium Alloys. Mater Trans. 2008;49(10):2170-8.

[30] Protivinsky J, Appleford M, Strnad J, Helebrant A, Ong JL. Effect of chemically modified titanium surfaces on protein adsorption and osteoblast precursor cell behavior. Int J Oral Maxillofac Implants. 2007;22(4):542-50.

[31] Hao L, Lawrence J. Wettability modification and the subsequent manipulation of protein adsorption on a Ti6Al4V alloy by means of CO2 laser surface treatment. J Mater Sci Mater Med. 2007;18(5):807-17.

[32] Cai K, Bossert J, Jandt KD. Does the nanometre scale topography of titanium influence protein adsorption and cell proliferation? Colloids Surf B Biointerfaces. 2006;49(2):136-44.

[33] Raghavendra S, Wood MC, Taylor TD. Early wound healing around endosseous implants: a review of the literature. Int J Oral Maxillofac Implants. 2005;20(3):425-31.

[34] Davies JE. Understanding peri-implant endosseous healing. J Dent Educ. 2003;67(8):932-49.

[35] Yang Y, Tian J, Deng L, Ong JL. Morphological behavior of osteoblast-like cells on surface-modified titanium in vitro. Biomaterials. 2002;23(5):1383-9.

[36] Zhao G, Raines AL, Wieland M, Schwartz Z, Boyan BD. Requirement for both micron- and submicron scale structure for synergistic responses of osteoblasts to substrate surface energy and topography. Biomaterials. 2007;28(18):2821-9.

[37] Watson PA. Function follows form: generation of intracellular signals by cell deformation. FASEB J. 1991;5(7):2013-9.

[38] Boyan BD, Batzer R, Kieswetter K, Liu Y, Cochran DL, Szmuckler-Moncler S, et al. Titanium surface roughness alters responsiveness of MG63 osteoblast-like cells to 1 alpha,25-(OH)2D3. J Biomed Mater Res. 1998;39(1):77-85.

[39] Lai HC, Zhuang LF, Liu X, Wieland M, Zhang ZY. The influence of surface energy on early adherent events of osteoblast on titanium substrates. J Biomed Mater Res A. 2009.

[40] Schwartz Z, Olivares-Navarrete R, Wieland M, Cochran DL, Boyan BD. Mechanisms regulating increased production of osteoprotegerin by osteoblasts cultured on microstructured titanium surfaces. Biomaterials. 2009;30(20):3390-6.

[41] Ong JL, Bess EG, Bessho K. Osteoblast progenitor cell responses to characterized titanium surfaces in the presence of bone morphogenetic protein-atelopeptide type I collagen in vitro. J Oral Implantol. 1999;25(2):95-100.

[42] Olivares-Navarrete R, Hyzy SL, Park JH, Dunn GR, Haithcock DA, Wasilewski CE, et al. Mediation of osteogenic differentiation of human mesenchymal stem cells on titanium surfaces by a Wnt-integrin feedback loop. Biomaterials. 2011;32(27):6399-411.

[43] Raines AL, Olivares-Navarrete R, Wieland M, Cochran DL, Schwartz Z, Boyan BD. Regulation of angiogenesis during osseointegration by titanium surface microstructure and energy. Biomaterials. 2010;31(18):4909-17.

[44] Olivares-Navarrete R, Hyzy SL, Hutton DL, Dunn GR, Appert C, Boyan BD, et al. Role of non-canonical Wnt signaling in osteoblast maturation on microstructured titanium surfaces. Acta Biomater. 2011;7(6):2740-50.

[45] Olivares-Navarrete R, Hyzy S, Wieland M, Boyan BD, Schwartz Z. The roles of Wnt signaling modulators Dickkopf-1 (Dkk1) and Dickkopf-2 (Dkk2) and cell maturation state in osteogenesis on microstructured titanium surfaces. Biomaterials. 2010;31(8):2015-24.

[46] Olivares-Navarrete R, Hyzy SL, Hutton DL, Erdman CP, Wieland M, Boyan BD, et al. Direct and indirect effects of microstructured titanium substrates on the induction of mesenchymal stem cell differentiation towards the osteoblast lineage. Biomaterials. 2010;31(10):2728-35.

[47] Cochran DL, Nummikoski PV, Higginbottom FL, Hermann JS, Makins SR, Buser D. Evaluation of an endosseous titanium implant with a sandblasted and acid-etched surface in the canine mandible: radiographic results. Clin Oral Implants Res. 1996;7(3):240-52.

[48] Cochran DL, Schenk RK, Lussi A, Higginbottom FL, Buser D. Bone response to unloaded and loaded titanium implants with a sandblasted and acid-etched

surface: a histometric study in the canine mandible. J Biomed Mater Res. 1998;40(1):1-11.

[49] Cochran DL, Buser D, ten Bruggenkate CM, Weingart D, Taylor TM, Bernard JP, et al. The use of reduced healing times on ITI implants with a sandblasted and acid-etched (SLA) surface: early results from clinical trials on ITI SLA implants. Clin Oral Implants Res. 2002;13(2):144-53.

[50] Bornstein MM, Harnisch H, Lussi A, Buser D. Clinical performance of wide-body implants with a sandblasted and acid-etched (SLA) surface: results of a 3-year follow-up study in a referral clinic. Int J Oral Maxillofac Implants. 2007;22(4):631-8.

[51] Schwartz Z, Raz P, Zhao G, Barak Y, Tauber M, Yao H, et al. Effect of micrometer-scale roughness of the surface of Ti6Al4V pedicle screws in vitro and in vivo. J Bone Joint Surg Am. 2008;90(11):2485-98.

[52] Bornstein MM, Hart CN, Halbritter SA, Morton D, Buser D. Early Loading of Nonsubmerged Titanium Implants with a Chemically Modified Sand-Blasted and Acid-Etched Surface: 6-Month Results of a Prospective Case Series Study in the Posterior Mandible Focusing on Peri-Implant Crestal Bone Changes and Implant Stability Quotient (ISQ) Values. Clin Implant Dent Relat Res. 2009.

[53] Marsell R, Einhorn TA. The biology of fracture healing. Injury. 2011;42(6):551-5.

[54] Brodbeck WG, Nakayama Y, Matsuda T, Colton E, Ziats NP, Anderson JM. Biomaterial surface chemistry dictates adherent monocyte/macrophage cytokine expression in vitro. Cytokine. 2002;18(6):311-9.

[55] Brodbeck WG, Patel J, Voskerician G, Christenson E, Shive MS, Nakayama Y, et al. Biomaterial adherent macrophage apoptosis is increased by hydrophilic and anionic substrates in vivo. Proc Natl Acad Sci U S A. 2002;99(16):10287-92.

[56] Kou PM, Schwartz Z, Boyan BD, Babensee JE. Dendritic cell responses to surface properties of clinical titanium surfaces. Acta Biomater. 2010.

[57] Sela J, Gross UM, Kohavi D, Shani J, Dean DD, Boyan BD, et al. Primary mineralization at the surfaces of implants. Crit Rev Oral Biol Med. 2000;11(4):423-36.

[58] Boyan BD, Lossdorfer S, Wang L, Zhao G, Lohmann CH, Cochran DL, et al. Osteoblasts generate an osteogenic microenvironment when grown on surfaces with rough microtopographies. Eur Cell Mater. 2003;6:22-7.

[59] Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys. 2008;473(2):139-46.

[60] Kobayashi Y, Udagawa N, Takahashi N. Action of RANKL and OPG for osteoclastogenesis. Crit Rev Eukaryot Gene Expr. 2009;19(1):61-72.

[61] Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews. 2008;14(2):179-86.

[62] Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, et al. The early fracture hematoma and its potential role in fracture healing. Tissue engineering Part B, Reviews. 2010;16(4):427-34.

[63] Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res. 2001;16(6):1004-14.

[64] Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res. 2002;17(3):513-20.

[65] Rundle CH, Wang H, Yu H, Chadwick RB, Davis EI, Wergedal JE, et al. Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. Bone. 2006;38(4):521-9.

[66] Isidro ML, Ruano B. Bone disease in diabetes. Current diabetes reviews. 2010;6(3):144-55.

[67] Duckers JM, Evans BA, Fraser WD, Stone MD, Bolton CE, Shale DJ. Low Bone Mineral Density in Men with Chronic Obstructive Pulmonary Disease. Respiratory research. 2011;12(1):101.

[68] Miller PD. Diagnosis and treatment of osteoporosis in chronic renal disease. Seminars in nephrology. 2009;29(2):144-55.

[69] Collier J. Bone disorders in chronic liver disease. Hepatology. 2007;46(4):1271-8.

[70] Donos N, Retzepi M, Wall I, Hamlet S, Ivanovski S. In vivo gene expression profile of guided bone regeneration associated with a microrough titanium surface. Clin Oral Implants Res. 2011;22(4):390-8.

[71] Ivanovski S, Hamlet S, Retzepi M, Wall I, Donos N. Transcriptional profiling of "guided bone regeneration" in a critical-size calvarial defect. Clin Oral Implants Res. 2011;22(4):382-9.

[72] Ivanovski S, Hamlet S, Salvi GE, Huynh-Ba G, Bosshardt DD, Lang NP, et al. Transcriptional profiling of osseointegration in humans. Clin Oral Implants Res. 2011;22(4):373-81.

[73] Terheyden H, Lang NP, Bierbaum S, Stadlinger B. Osseointegration – communication of cells. Clinical Oral Implants Research. 2011:n/a-n/a.

[74] Dinarello CA. Interleukin-1 and tumor necrosis factor: effector cytokines in autoimmune diseases. Semin Immunol. 1992;4(3):133-45.

[75] Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996;87(6):2095-147.

[76] Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature. 1986;324(6092):73-6.

[77] Kishimoto T. The biology of interleukin-6. Blood. 1989;74(1):1-10.

[78] Roodman GD. Regulation of osteoclast differentiation. Ann N Y Acad Sci. 2006;1068:100-9.

[79] Blanchard F, Duplomb L, Baud'huin M, Brounais B. The dual role of IL-6-type cytokines on bone remodeling and bone tumors. Cytokine Growth Factor Rev. 2009;20(1):19-28.

[80] Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol. 2007;25:193-219.

[81] Shen F, Ruddy MJ, Plamondon P, Gaffen SL. Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-alpha-induced genes in bone cells. J Leukoc Biol. 2005;77(3):388-99.

[82] Gaffen SL. Biology of recently discovered cytokines: interleukin-17--a unique inflammatory cytokine with roles in bone biology and arthritis. Arthritis Res Ther. 2004;6(6):240-7.

[83] Miljkovic D, Trajkovic V. Inducible nitric oxide synthase activation by interleukin-17. Cytokine Growth Factor Rev. 2004;15(1):21-32.

[84] de Vries JE. Immunosuppressive and anti-inflammatory properties of interleukin 10. Ann Med. 1995;27(5):537-41.

[85] Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine Growth Factor Rev. 2010;21(5):331-44.

[86] Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest. 2000;117(4):1162-72.

[87] Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors. 2004;22(4):233-41.

[88] Thorey F, Menzel H, Lorenz C, Gross G, Hoffmann A, Windhagen H. Osseointegration by bone morphogenetic protein-2 and transforming growth factor beta2 coated titanium implants in femora of New Zealand white rabbits. Indian journal of orthopaedics. 2011;45(1):57-62.

[89] Decker JF, Lee J, Cortella CA, Polimeni G, Rohrer MD, Wozney JM, et al. Evaluation of implants coated with recombinant human bone morphogenetic protein-2 and vacuum-dried using the critical-size supraalveolar peri-implant defect model in dogs. J Periodontol. 2010;81(12):1839-49.

[90] Bishop GB, Einhorn TA. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop. 2007;31(6):721-7.

[91] Garrison KR, Donell S, Ryder J, Shemilt I, Mugford M, Harvey I, et al. Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. Health technology assessment. 2007;11(30):1-150, iii-iv.

[92] Burkus JK, Dorchak JD, Sanders DL. Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine (Phila Pa 1976). 2003;28(4):372-7.

[93] Glassman SD, Dimar JR, Carreon LY, Campbell MJ, Puno RM, Johnson JR. Initial fusion rates with recombinant human bone morphogenetic protein-2/compression resistant matrix and a hydroxyapatite and tricalcium phosphate/collagen carrier in posterolateral spinal fusion. Spine (Phila Pa 1976). 2005;30(15):1694-8.

[94] Boden SD, Kang J, Sandhu H, Heller JG. Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial: 2002 Volvo Award in clinical studies. Spine (Phila Pa 1976). 2002;27(23):2662-73.

[95] Mindea SA, Shih P, Song JK. Recombinant human bone morphogenetic protein-2-induced radiculitis in elective minimally invasive transforaminal lumbar interbody fusions: a series review. Spine (Phila Pa 1976). 2009;34(14):1480-4; discussion 5.

[96] Leknes KN, Yang J, Qahash M, Polimeni G, Susin C, Wikesjo UM. Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2: radiographic observations. Clin Oral Implants Res. 2008;19(10):1027-33.

[97] Rupp F, Scheideler L, Olshanska N, deWild M, Wieland M, Geis-Gerstorfer J. Enhancing surface free energy and hydrophilicity through chemical modification of microstructured titanium implant surfaces. Journal of Biomedical Materials. 2006;76A:323-34.

[98] Zhao G, Schwartz Z, Wieland M, Rupp F, Geis-Gerstorfer J, Cochran DL, et al. High surface energy enhances cell response to titanium substrate microstructure. J Biomed Mater Res A. 2005;74(1):49-58.

[99] Rausch-fan X, Qu Z, Wieland M, Matejka M, Schedle A. Differentiation and cytokine synthesis of human alveolar osteoblasts compared to osteoblast-like cells (MG63) in response to titanium surfaces. Dent Mater. 2008;24(1):102-10.

[100] Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J, Jr., et al. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). J Biomed Mater Res. 1995;29(3):389-401.

[101] Choo MK, Kawasaki N, Singhirunnusorn P, Koizumi K, Sato S, Akira S, et al. Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade. Mol Cancer Ther. 2006;5(12):2970-6.

[102] Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, et al. A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. J Biol Chem. 2003;278(20):18485-90.

[103] Hong CC, Yu PB. Applications of small molecule BMP inhibitors in physiology and disease. Cytokine Growth Factor Rev. 2009;20(5-6):409-18.

[104] Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol. 2008;4(1):33-41.

[105] Hao J, Daleo MA, Murphy CK, Yu PB, Ho JN, Hu J, et al. Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. PLoS One. 2008;3(8):e2904.

[106] Hidaka H, Inagaki M, Kawamoto S, Sasaki Y. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry. 1984;23(21):5036-41.

[107] Gao Y, Dhanakoti S, Tolsa JF, Raj JU. Role of protein kinase G in nitric oxide- and cGMP-induced relaxation of newborn ovine pulmonary veins. J Appl Physiol. 1999;87(3):993-8.

[108] Niisato N, Ito Y, Marunaka Y. Effects of PKA inhibitors, H-compounds, on epithelial Na+ channels via PKA-independent mechanisms. Life Sci. 1999;65(10):PL109-14.

[109] Ding J, Ghali O, Lencel P, Broux O, Chauveau C, Devedjian JC, et al. TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. Life Sci. 2009;84(15-16):499-504.

[110] Lange J, Sapozhnikova A, Lu C, Hu D, Li X, Miclau T, 3rd, et al. Action of IL-1beta during fracture healing. J Orthop Res. 2010;28(6):778-84.

[111] Miclau T, Lu C, Thompson Z, Choi P, Puttlitz C, Marcucio R, et al. Effects of delayed stabilization on fracture healing. J Orthop Res. 2007;25(12):1552-8.

[112] Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med. 2000;51:245-70.

[113] Taki N, Tatro J, Lowe R, Goldberg V, Greenfield E. Comparison of the roles of IL-1, IL-6, and TNFα in cell culture and murine models of aseptic loosening. Bone. 2007;40(5):1276-83.

[114] Stea S, Visentin M, Granchi D, Ciapetti G, Donati ME, Sudanese A, et al. Cytokines and osteolysis around total hip prostheses. Cytokine. 2000;12(10):1575-9.

[115] Konttinen YT, Xu JW, Waris E, Li TF, Gomez-Barrena E, Nordsletten L, et al. Interleukin-6 in aseptic loosening of total hip replacement prostheses. Clinical and experimental rheumatology. 2002;20(4):485-90.

[116] Petkovic AB, Matic SM, Stamatovic NV, Vojvodic DV, Todorovic TM, Lazic ZR, et al. Proinflammatory cytokines (IL-1beta and TNF-alpha) and chemokines (IL-8 and MIP-1alpha) as markers of peri-implant tissue condition. Int J Oral Maxillofac Surg. 2010;39(5):478-85.

[117] Akech J, Wixted JJ, Bedard K, van der Deen M, Hussain S, Guise TA, et al. Runx2 association with progression of prostate cancer in patients: mechanisms mediating bone osteolysis and osteoblastic metastatic lesions. Oncogene. 2010;29(6):811-21.

[118] Lubberts E, van den Bersselaar L, Oppers-Walgreen B, Schwarzenberger P, Coenen-de Roo CJ, Kolls JK, et al. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. J Immunol. 2003;170(5):2655-62.

[119] Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun. 2000;275(3):768-75.

[120] Brandtzaeg P, Osnes L, Ovstebo R, Joo GB, Westvik AB, Kierulf P. Net inflammatory capacity of human septic shock plasma evaluated by a monocyte-based target cell assay: identification of interleukin-10 as a major functional deactivator of human monocytes. J Exp Med. 1996;184(1):51-60.

[121] Clarke CJ, Hales A, Hunt A, Foxwell BM. IL-10-mediated suppression of TNF-alpha production is independent of its ability to inhibit NF kappa B activity. Eur J Immunol. 1998;28(5):1719-26.

[122] Hamlet S, Alfarsi M, George R, Ivanovski S. The effect of hydrophilic titanium surface modification on macrophage inflammatory cytokine gene expression. Clinical Oral Implants Research. 2011:n/a-n/a.

[123] Hamlet S, Ivanovski S. Inflammatory cytokine response to titanium chemical composition and nanoscale calcium phosphate surface modification. Acta Biomater. 2011;7(5):2345-53.

[124] Takebe J, Champagne CM, Offenbacher S, Ishibashi K, Cooper LF. Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line. J Biomed Mater Res A. 2003;64(2):207-16.

[125] Refai AK, Textor M, Brunette DM, Waterfield JD. Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. J Biomed Mater Res A. 2004;70(2):194-205.

[126] Smoljanovic T, Cimic M, Bojanic I. Aggressive end plate decortication as a cause of osteolysis after rhBMP-2 use in cervical spine interbody fusion. Spine J. 2010;10(2):187-8; author reply 8.

[127] Lee KB, Murray SS, Taghavi CE, Song KJ, Brochmann EJ, Johnson JS, et al. Bone morphogenetic protein-binding peptide reduces the inflammatory response to recombinant human bone morphogenetic protein-2 and recombinant human bone morphogenetic protein-7 in a rodent model of soft-tissue inflammation. Spine J. 2011;11(6):568-76.

[128] Lee KB, Taghavi CE, Song KJ, Sintuu C, Yoo JH, Keorochana G, et al. Inflammatory characteristics of rhBMP-2 in vitro and in an in vivo rodent model. Spine (Phila Pa 1976). 2011;36(3):E149-54.

[129] Akeel S, El-Awady A, Hussein K, El-Refaey M, Elsalanty M, Sharawy M, et al. Recombinant bone morphogenetic protein-2 induces up-regulation of vascular endothelial growth factor and interleukin 6 in human pre-osteoblasts: Role of reactive oxygen species. Arch Oral Biol. 2011.

[130] Fukuno N, Matsui H, Kanda Y, Suzuki O, Matsumoto K, Sasaki K, et al. TGF-beta-activated kinase 1 mediates mechanical stress-induced IL-6 expression in osteoblasts. Biochem Biophys Res Commun. 2011;408(2):202-7.

[131] Courties G, Seiffart V, Presumey J, Escriou V, Scherman D, Zwerina J, et al. In vivo RNAi-mediated silencing of TAK1 decreases inflammatory Th1 and Th17 cells through targeting of myeloid cells. Blood. 2010;116(18):3505-16.

[132] Urist MR. Bone: formation by autoinduction. Science. 1965;150(698):893-9.

[133] Reddi AH, Huggins C. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci U S A. 1972;69(6):1601-5.

[134] Vukicevic S, Sampath KT. Bone morphogenetic proteins : regeneration of bone and beyond. Basel: Birkha\0308user; 2004. xi, 310 p. p.

[135] Lewandrowski KU, Nanson C, Calderon R. Vertebral osteolysis after posterior interbody lumbar fusion with recombinant human bone morphogenetic protein 2: a report of five cases. Spine J. 2007;7(5):609-14.

[136] ten Dijke P, Korchynskyi O, Valdimarsdottir G, Goumans MJ. Controlling cell fate by bone morphogenetic protein receptors. Mol Cell Endocrinol. 2003;211(1-2):105-13.

[137] Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. Cell. 1998;95(6):737-40.

[138] Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, et al. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science. 1996;272(5265):1179-82.

[139] Shirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, et al. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. J Biol Chem. 1997;272(13):8141-4.

[140] Lee YS, Chuong CM. Activation of protein kinase A is a pivotal step involved in both BMP-2- and cyclic AMP-induced chondrogenesis. J Cell Physiol. 1997;170(2):153-65.

[141] Zhao L, Yang S, Zhou GQ, Yang J, Ji D, Sabatakos G, et al. Downregulation of cAMP-dependent protein kinase inhibitor gamma is required for BMP-2-induced osteoblastic differentiation. Int J Biochem Cell Biol. 2006;38(12):2064-73.

[142] Walsh DW, Godson C, Brazil DP, Martin F. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol. 2010;20(5):244-56.

[143] Wagner DO, Sieber C, Bhushan R, Borgermann JH, Graf D, Knaus P. BMPs: from bone to body morphogenetic proteins. Sci Signal. 2010;3(107):mr1.

[144] Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 1996;10(13):1580-94.

[145] Marazzi G, Wang Y, Sassoon D. Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. Dev Biol. 1997;186(2):127-38.

[146] Zhang J, Ge Y, Sun L, Cao J, Wu Q, Guo L, et al. Effect of bone morphogenetic protein-2 on proliferation and apoptosis of gastric cancer cells. International journal of medical sciences. 2012;9(2):184-92.

[147] Kawamura C, Kizaki M, Ikeda Y. Bone morphogenetic protein (BMP)-2 induces apoptosis in human myeloma cells. Leuk Lymphoma. 2002;43(3):635-9.

[148] Hay E, Lemonnier J, Fromigue O, Marie PJ. Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. J Biol Chem. 2001;276(31):29028-36.

[149] Pajni-Underwood S, Wilson CP, Elder C, Mishina Y, Lewandoski M. BMP signals control limb bud interdigital programmed cell death by regulating FGF signaling. Development. 2007;134(12):2359-68.

[150] Yokouchi Y, Sakiyama J, Kameda T, Iba H, Suzuki A, Ueno N, et al. BMP-2/-4 mediate programmed cell death in chicken limb buds. Development. 1996;122(12):3725-34.

[151] Rosado E, Schwartz Z, Sylvia VL, Dean DD, Boyan BD. Transforming growth factor-beta1 regulation of growth zone chondrocytes is mediated by multiple interacting pathways. Biochim Biophys Acta. 2002;1590(1-3):1-15.

[152] Ryoo HM, Lee MH, Kim YJ. Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. Gene. 2006;366(1):51-7.

[153] Edgar CM, Chakravarthy V, Barnes G, Kakar S, Gerstenfeld LC, Einhorn TA. Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. Bone. 2007;40(5):1389-98.

[154] Solmesky LJ, Abekasis M, Bulvik S, Weil M. Bone morphogenetic protein signaling is involved in human mesenchymal stem cell survival in serum-free medium. Stem Cells Dev. 2009;18(9):1283-92.

[155] Hay E, Lemonnier J, Fromigue O, Guenou H, Marie PJ. Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. J Biol Chem. 2004;279(3):1650-8.

[156] Kawabata M, Imamura T, Miyazono K. Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev. 1998;9(1):49-61.

[157] He F, Xiong W, Wang Y, Matsui M, Yu X, Chai Y, et al. Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis. Dev Biol. 2010;347(1):109-21.

[158] Sharov AA, Weiner L, Sharova TY, Siebenhaar F, Atoyan R, Reginato AM, et al. Noggin overexpression inhibits eyelid opening by altering epidermal apoptosis and differentiation. EMBO J. 2003;22(12):2992-3003.

[159] Anderson RM, Stottmann RW, Choi M, Klingensmith J. Endogenous bone morphogenetic protein antagonists regulate mammalian neural crest generation and survival. Dev Dyn. 2006;235(9):2507-20.

[160] Sutherland MK, Geoghegan JC, Yu C, Turcott E, Skonier JE, Winkler DG, et al. Sclerostin promotes the apoptosis of human osteoblastic cells: a novel regulation of bone formation. Bone. 2004;35(4):828-35.

[161] Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development. 1999;126(23):5515-22.

[162] Fajardo M, Liu CJ, Egol K. Levels of expression for BMP-7 and several BMP antagonists may play an integral role in a fracture nonunion: a pilot study. Clin Orthop Relat Res. 2009;467(12):3071-8.

[163] Chang SC, Chuang HL, Chen YR, Chen JK, Chung HY, Lu YL, et al. Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. Gene therapy. 2003;10(24):2013-9.

[164] Beck SE, Jung BH, Del Rosario E, Gomez J, Carethers JM. BMP-induced growth suppression in colon cancer cells is mediated by p21WAF1 stabilization and modulated by RAS/ERK. Cell Signal. 2007;19(7):1465-72.

[165] Cannon JE, Upton PD, Smith JC, Morrell NW. Intersegmental vessel formation in zebrafish: requirement for VEGF but not BMP signalling revealed by selective and non-selective BMP antagonists. British journal of pharmacology. 2010;161(1):140-9.

[166] Rath B, Nam J, Deschner J, Schaumburger J, Tingart M, Grassel S, et al. Biomechanical forces exert anabolic effects on osteoblasts by activation of SMAD 1/5/8 through type 1 BMP receptor. Biorheology. 2011;48(1):37-48.

[167] Vogt J, Traynor R, Sapkota GP. The specificities of small molecule inhibitors of the TGFss and BMP pathways. Cell Signal. 2011;23(11):1831-42.

[168] Boergermann JH, Kopf J, Yu PB, Knaus P. Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells. Int J Biochem Cell Biol. 2010;42(11):1802-7.